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**Translationally relevant mouse model of early life cancer and chemotherapy exposure
results in brain and small intestine cytokine responses: a potential link to cognitive
deficits**

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Abstract

Survivors of acute lymphoblastic leukemia (ALL), the most common childhood cancer, are at increased risk for long-term cognitive problems, including executive function deficits. The chemotherapeutic agent methotrexate (MTX) is used to treat most ALL patients, and is closely associated with cognitive deficits. To address how early life cancer chemotherapy leads to cognitive deficits, we developed a translationally relevant mouse model of leukemia survival that exposed mice to leukemic cells and chemotherapeutic drugs (vincristine and MTX, with leucovorin rescue) in early life. Male and female mice were tested several weeks later using novel object recognition (recognition memory) and 5-choice serial reaction time task (executive function). Gene expression of proinflammatory, white matter and synapse-associated molecules was assessed in the prefrontal cortex and small intestine both acutely after chemotherapy and chronically after cognitive testing. Early life cancer-chemotherapy exposure resulted in recognition memory and executive function deficits in adult male mice. Prefrontal cortex expression of the chemokine *Ccl2* was increased acutely, while small intestine expression of the proinflammatory cytokine tumor necrosis factor-alpha was elevated both acutely (both sexes) and chronically (males only). Inflammation in the small intestine was correlated with prefrontal cortical proinflammatory and synaptic gene expression changes, as well as to executive function deficits. Collectively, these data indicate that the current protocol results in a robust mouse model in which to study cognitive deficits in leukemia survivors, and suggest that small intestine inflammation may represent a novel contributor to adverse CNS consequences of early life chemotherapy.

Keywords: chemotherapy, executive function, small intestine inflammation, prefrontal cortex

Introduction

Acute Lymphoblastic Leukemia (ALL) is the most common childhood cancer (Howlader et al., 2013), with a survival rate of nearly 90% (Hunger and Mullighan, 2015). However, even in the absence of cranial irradiation, childhood ALL survivors often suffer from various adverse effects of chemotherapy including executive function and memory deficits as well as emotional impairments, such as anxiety, depression, and social withdrawal (Insel et al., 2017; Iyer et al., 2015; Jacola et al., 2016a; Jacola et al., 2016b; Kanellopoulos et al., 2016; Kunin-Batson et al., 2016; Liu et al., 2018; Myers et al., 2014; Phillips et al., 2020; van der Plas et al., 2017; Zhou et al., 2020).

The prevalence of neurocognitive problems in survivors of childhood cancer treated with chemotherapy is estimated to be 20-40% and dose-dependently related to systemically-administered methotrexate (MTX) (Jacola et al., 2016a; Krull et al., 2013), a widely used chemotherapeutic drug for ALL. Experimental studies have also shown long-term cognitive deficits after MTX injection in juvenile rodents and reported compromised dendritic architecture, altered white matter and effects on microglia in both gray and white matter (Alexander et al., 2018; Gibson et al., 2019; Li et al., 2010; Wen et al., 2018).

However, these studies have assessed effects of MTX in healthy, tumor-free animals, and clinical studies have shown that encephalopathy can be present in children at ALL diagnosis, indicating that cancer has independent adverse effects on the brain prior to subsequent aggravation by chemotherapy (Korinthenberg et al., 2002). Moreover, it has been shown both in adult cancer patients and tumor-bearing animals that affective and cognitive changes can be present before any chemotherapeutic treatment (Schrepf et al., 2015). Taken together, these findings indicate the importance of incorporating both cancer and chemotherapy into animal models designed to investigate candidate mechanisms mediating the lasting consequences of early life chemotherapy on cognition and behavior.

Therefore, in the present work we developed a model of early life cancer and chemotherapy exposure to evaluate long-term cognitive sequelae. As the clinical literature indicates that executive function is particularly sensitive to long-term effects of childhood ALL chemotherapy (Cheung and Krull, 2015; Jacola et al., 2016b; van der Plas et al., 2017), we used advanced operant testing to assess executive function in this animal model. Prefrontal cortical expression of cytokines, white matter related genes and synaptic markers at both acute (immediately following chemotherapy) and long-term (after behavioral testing) time points were measured. Adverse CNS consequences of cancer chemotherapy may also depend on peripheral inflammatory processes (Bajic et al., 2018; Wang et al., 2015; Wardill et al., 2016) with chemotherapy affecting small intestine function, including secretion, motility and barrier properties (Branski et al., 1981; Lifschitz and Mahoney, 1989; Pal, 1999; Pearson et al., 1984; Sninsky, 1987; Vera et al., 2017; Wardill et al., 2012). In addition, these drugs induce morphological abnormalities in the small intestine accompanied by nitrosative stress and neutrophil infiltration (Bero and Javor, 1985; Gwavava et al., 1981; Iqbal et al., 2001; Jellinghaus et al., 1977; Kolli et al., 2008; Nazir et al., 2017; Pinkerton et al., 1982; Vera et al., 2017). The loss of small intestinal barrier properties and ensuing bacterial translocation from gut to plasma along with cell necrosis can explain the occurrence of intestinal inflammation with increased production of pro-inflammatory cytokines, inflammation of alimentary tract mucosa (mucositis) after MTX administration (Mazur et al., 2004; Morales-Rojas et al., 2012; Van Sebille et al., 2015). Interestingly, folinic acid administration (leucovorin) cannot prevent MTX-induced gut permeability and translocation of bacterial fragments (Meng et al., 2016) and increased production of pro-inflammatory cytokines, inflammation of alimentary tract mucosa (mucositis) (Mazur et al., 2004; Morales-Rojas et al., 2012). Furthermore, vincristine (VCR), often used along with MTX, results in vagal neuropathy (Weissman-Fogel et al., 2008), which may further promote inflammation through

reduced activation of the vagal anti-inflammatory pathway (Cailotto et al., 2014; Matteoli et al., 2014; Murray et al., 2019). Therefore, expression of cytokines and tight junction proteins was also studied both acutely and long-term in the small intestine.

Materials and methods

Animals

B6D2F1 hybrids were generated through in house breeding of C57BL/6J females and DBA/2J males (Jackson Laboratories, Bar Harbor ME). Animals were fed an ad-lib diet (Teklad LM-485, Madison WI) and maintained on a 12 hr light – dark cycle in standard microisolator cages. Lights were on from 07.00 till 19.00 h, then switched to a reverse light-dark cycle for behavioral experiments (see details below). Upon weaning at PN21 animals were grouped housed 3-4 mice per cage. All animals were cared for according to the guidelines of the University of Cincinnati Institutional Animal Care and Use Committee.

Leukemia cell preparation and injection

L1210 cells (CCL-219; ATCC, Manassas, VA, USA) were grown *in vitro* according to the distributor's recommendations. L1210 cells were chosen because they are autologous in C57BL6/DBA mice, result in a growing tumor with a predictable lifecycle, and have been used to identify chemotherapeutic agents that are effective against leukemia (Johnson and Hardy, 1965; Skipper et al., 1964; Venditti and Goldin, 1964). Nineteen day old mice were injected subcutaneously between the scapulae with 10^3 , 10^4 or 10^6 freshly-harvested L1210 cells or its sterile phosphate-buffered saline (PBS) vehicle (0.1 ml).

Chemotherapy

Beginning two days after administration of L1210 cells, vincristine, (VCR; 0.5 mg/kg; Hospira, Lake Forest, IL, USA), MTX (50 mg/kg; Hospira, Lake Forest, IL, USA) and leucovorin (2 mg/kg; LE117 Spectrum, New Brunswick NJ, USA), all dissolved in sterile saline (Hospira, Lake Forest, IL, USA), were injected intraperitoneally every four days for four cycles (see Fig S1 for timeline). The sequence and timing of drug administration was

VCR at 1600, followed 30 minutes later by MTX, and 16 hrs later by leucovorin. Control mice received saline vehicle injections. Cognitive side effects have been linked more strongly to MTX than to vincristine, which does not cross the blood brain barrier, however, vincristine is necessary for long term survival. Leucovorin (folinic acid), a folate rescue therapy, is administered to allow for higher dose MTX administration. Vincristine is a vinca alkaloid and acts as an antimicrotubule agent that blocks mitosis by arresting cells in the metaphase, while methotrexate inhibits thymidine synthesis (and subsequently DNA / RNA synthesis and cell proliferation) by interrupting the conversion of folate into the active metabolite THF via blockade of dihydrofolate reductase. However, beyond the intended inhibition of cancer cell growth, MTX also acts on healthy cells. In an effort to minimize side effects, folinic acid (leucovorin) has been incorporated into contemporary chemotherapy protocols because it does not require DHFR for conversion to active THF.

Post-leukemia-chemotherapy monitoring

After completion of chemotherapy, body weight was measured every other day and tumor size was measured every 2-3 days upon first identification. Any animal with a body conditioning score ≤ 2 (Ullman-Cullere and Foltz, 1999), having lost more than 20% of bodyweight or showing a sudden increase in body weight (reflecting end-stage tumor growth) was terminated by cervical dislocation.

Behavioral testing

To ensure mice were tested during their active phase, one week prior to the onset of behavioral testing, animals were switched to a reverse light-dark cycle, with lights off at 09.00 h, and on at 21.00 h. Behavioral experiments were initiated at 10.00 h. Social interaction was

assessed at six weeks of age (3 weeks after chemotherapy started) after introduction of a conspecific mouse into the home cage of the experimental mice. Open field behavior (locomotor and exploratory behavior) and novel object recognition (recognition memory) were completed at 9-10 weeks of age (4-5 weeks after chemotherapy). Details of the behavioral assays are located in the supplemental methods.

Operant behavior. At 11 weeks of age (8 weeks after the start of chemotherapy), animals were food-restricted to 85-90% of their free feeding body weight over the course of a week and animals were pre-exposed to the reinforcer (Yoo-hoo, Dr. Pepper Snapple Group Inc., Plano, TX, USA) for 24 hours in the home cage. Operant testing was carried out in Bussey-Saksida Mouse Touch Screen operant chambers (Lafayette Instrument, Lafayette, IN, USA) running ABET II Touch software as previously described (Makinson et al., 2019; Smith et al., 2020). Basic cognition was assessed using a fixed ratio (FR) 1 schedule, and motivation was assessed using a progressive ratio (PR) schedule. Following FR1 and PR, executive function was assessed.

Five choice serial reaction time task and titration schedule. Animals that met FR1 criterion (>70 correct trials in two consecutive sessions) continued onto 5 choice serial reaction (5-CSRTT) training, as previously described (Grissom et al., 2014; Smith et al., 2020), described in detail in supplemental methods. Briefly, animals were required to touch one of 5 illuminated areas for completion of a correct trial. A premature trial was recorded if an animal made a response during the intertrial interval (ITI). If an animal responded to an unilluminated area during stimulus presentation, then an incorrect trial was recorded. If no response was made, an omitted trial was recorded. After the 5CSRTT, the animals moved on to the titration schedule, which incorporates a variable ITI to assess attentional capacity.

Tissue preparation. Animals were sacrificed via cervical dislocation for PCR mRNA analyses 24 h after the last MTX injection (acute; PN34) or one week after completion of titration (chronic; PN152-154). Intestine was gently flushed with cold sterile saline, and dissected into 6 sections; duodenum, proximal jejunum, distal jejunum, ileum, proximal colon and distal colon and placed in to RNA later. For small intestine analysis, an ~0.5 cm section from the duodenum, proximal and distal jejunum was pooled. Whole brains and small intestine were placed in RNAlater (Thermo Fisher Scientific) and stored at -20 °C. For immunohistochemical analysis, another cohort of animals were deeply anesthetized and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde (pH 7.4) at PN34.

Polymerase Chain Reaction. The medial prefrontal cortex was dissected from a 2 mm coronal slice from bregma +2.3 to 0.3 and RNA was extracted using the Trizol protocol. cDNA was synthesized using the High Capacity Reverse Transcriptase kit (Applied Biosystems). Gene expression was measured via qPCR on a 7900HT Fast Real-Time PCR System using Taqman primers and Taqman Gene Expression Master Mix. (Table 1 for gene list). Expression of targets was normalized to the geomean of the housekeeping genes *actb* and *gapdh*, expressed as relative quantification values, and analyzed by two-way ANOVA with the factors sex and treatment.

Immunohistochemical localization of Iba1. Iba1 expression was used to identify microglia following our published protocol (Makinson et al., 2017). Briefly, thirty- μ m brain sections were incubated overnight at 4°C in primary antibody (1:2000 IBA1, Wako, 019-19741), and subsequently with biotinylated goat antirabbit secondary antibody (Vector Laboratories, Burlingame, CA, BA-1000). Immunoreactivity was visualized with 3,3'-diaminobenzine.

Images were collected at 10x for counts and 40x for perimeter and area analysis. For counts, templates were created in Photoshop (Adobe Photoshop, Adobe Systems, San Jose, CA, USA) and overlaid on each image to ensure equal tissue area was included in each count. Cell counts, perimeter and area measurements were performed in ImageJ (<http://imagej.nih.gov/ij/>; additional details in supplemental methods).

Data analysis

Two-way ANOVA (cancer-chemotherapy x sex), unless otherwise indicated, was used to evaluate results using GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA). Repeated-measures three-way ANOVA was employed to analyze body weight and Fixed Ratio 1 training data. Bonferroni-corrected post hoc planned comparisons were conducted when appropriate. The comparisons of interest were; (1) control versus cancer-chemotherapy and (2) female versus male. Interactions and main effects are described in the results and significant post hoc comparisons are indicated in the figures, with $p < 0.05$ considered significant. Exploratory correlation analyses were conducted to determine (1) whether gene expression changes in the brain and small intestine were related, and (2) whether gene expression changes were related to cognitive performance in the 5CSRTT. As these analyses were exploratory, no correction for multiple comparisons was applied (Armstrong, 2014).

Results

Comparison of L1210 doses on survival. In an initial pilot experiment, saline, 10^4 or 10^6 L1210 cells were injected at P19 into male (n=11, 13, 14, respectively) and female (n=11, 18, 14, respectively) animals, and the chemotherapy protocol was completed as described (Fig S1). With 10^6 L1210 cells, all animals developed measurable tumors and no animal survived beyond 25 (male) or 28 (female) days (Fig S2). With 10^4 L1210 cells, long-term survival rates remained below 50%. Therefore a second experiment was conducted comparing saline injections to 10^3 L1210 cells (male n= 13, 19; female 12, 15, respectively). There were no deaths prior to d36 post-injection, and the long-term survival rate (>128 days) was 89% in males and 83% in females. Tumors were evident in 2/19 males and 3/15 females, and were first detected on days 25-31 post-L1210 injection. Animals with tumors were humanely euthanized after the tumor was first identified.

Body weight data. Three-way ANOVA (treatment x sex x time) revealed a 3-way interaction ($F(13, 754) = 2.833, p=0.0005$). Posthoc analyses indicated that cancer-chemotherapy reduced body weight gain in males between PND 31-43, while bodyweights in females did not differ significantly (Fig S3).

Early cancer-chemotherapy exposure reduced social interaction but did not durably affect exploratory behavior

Eleven to twelve days following the final chemotherapy treatment, social interaction was assessed. The mean approach duration was significantly lower in mice exposed to cancer-chemotherapy (Fig 1A; Main effect treatment; $F(1, 47)=10.8, p<0.01$) and significantly higher in females than in males (main effect sex; $F(1, 47)=11.4, p<0.01$), with no significant interaction occurring between treatment and sex. Four to five weeks after conclusion of

chemotherapy, mice were tested in the open field. Time spent in the center, speed or distance traveled of the brightly-lit open field were not found to be different between treatments or sexes. Similarly, locomotor activity registered as beam breaks did not differ between groups during operant testing (data not shown).

Early cancer-chemotherapy exposure impaired novel object recognition in adult males

All animals were able to discriminate between a novel and familiar object and spent significantly more time with the novel object (discrimination index significantly above 0.5, $t_{12}=7.11$, $t_{15}=9.76$, t_{11} , 10.69 , $t_{14}=9.83$, all $p<0.0001$; male control, male cancer-chemo, female control, female cancer-chemo, respectively). However, cancer-chemotherapy exposed animals discriminated less well between the novel and familiar object as compared to control animals (Fig 1B; $F(1, 52)=4.36$, $p<0.05$), an effect driven by male animals, as posthoc comparisons identified a significant difference between male control and treated animals ($t_{29}=2.69$, $p<0.05$).

Early cancer-chemotherapy accelerated acquisition of operant behavior

Criterion for acquisition of FR1 responding was successful completion of 70 trials within a 30 min testing session. In this task, cancer-chemotherapy exposed animals reached criterion in significantly fewer days than controls (Fig S4A; approximately one day earlier; main effect treatment; $F(1, 52)=4.96$, $p<0.05$). Females were also faster to learn this task (main effect sex $F(1, 52)=20.6$, $p<0.0001$). In PR, no significant differences in performance were observed between experimental groups.

Early cancer-chemotherapy altered adult five choice serial reaction performance

To assess executive function, animals were tested in the 5CSRTT. Initially, animals progressed through three increasingly difficult levels, as the stimulus duration was progressively shortened (12, 8, 4 sec). In the first step of the 5CSRTT (12 sec stimulus), analysis of percent premature responding revealed a significant interaction ($F(1, 52)=6.64$, $p<0.05$), such that male cancer-chemotherapy animals had significantly more premature responses than controls ($t_{29}=2.74$, $p<0.01$), while female animals did not differ (Fig 1C). There were no differences between groups in incorrect or omitted responses. As testing progressed with trials at shorter stimulus lengths (8 and 4 sec) and as animals gained familiarity with the task, no group differences were evident.

The final stage of the 5CSRTT was titration and involved establishing the minimum stimulus length at which the animal can still make a correct response. The median stimulus length was lower in mice that had been subject to cancer-chemotherapy treatment (Fig S4B; $F(1, 52)=7.72$ $p<0.01$) as well as females ($F(1, 52)=4.15$ $p<0.05$). Similarly, the shortest stimulus length at which animals still made correct responses was found to be significantly lower in cancer-chemotherapy animals as compared to controls ($F(1, 52)=5.62$ $p<0.05$, Fig S5A). Additionally, females were found to complete significantly more trials, and to make significantly more premature responses than males during the 30 min session ($F(1, 52) = 7.60$, $p<0.01$ and $F(1, 47) = 18.53$, $p<0.0001$, Fig S5B,C).

Early cancer-chemotherapy reduced brain weight and altered cortical gene expression acutely

Two-way ANOVA revealed that one day after the final MTX/VCR administration, differences in brain weight (corrected by body weight) were dependent on sex (interaction; $F(1, 30)=13.30$, $p<0.01$), such that relative brain weight was lower in male mice that

underwent cancer-chemotherapy (Fig 2A; $t_{18}=3.35$, $p<0.01$) and not different in females. In the prefrontal cortex of these brains, mRNA expression of white matter myelin-binding protein and oligodendrocyte transcription factor 2 (Fig 2B,C), but not that of myelin oligodendrocyte glycoprotein and platelet-derived growth factor receptor alpha, were found to be significantly lower in animals that were subject to cancer-chemotherapy exposure as compared to mice that were not (main effect of treatment; $F(1, 20)=7.81$, $p<0.05$, $F(1, 20)=4.90$, $p<0.05$, respectively). Analysis of immune-related transcripts, identified a significant interaction between treatment and sex for *Ccl2* expression (Fig 3A; $F(1, 32) = 6.32$, $p<0.05$), and posthoc analysis revealed that treatment led to an increase in *Ccl2* in males ($t_{15}=2.71$, $p<0.05$), but not females. There were no differences in *Il-1 β* , *Cr3*, *Tnfa*, *Cxcl10*, *Dnmt1*, *Syp*, *Gphn* or *Psd95*, and there were no differences in the number of microglia or the perimeter or area of microglia within PFC (Fig S6).

Early cancer-chemotherapy increased intestinal expression of claudin-1 and tumor necrosis factor alpha acutely

One day after the final MTX/VCR administration, expression of tight junction-associated and inflammation-related molecules were examined in the intestine. Two-way ANOVA analysis of claudin-1 expression revealed a significant interaction of treatment and sex ($F(1, 32)=4.19$, $p<0.05$), with main effects of both sex and treatment ($F(1, 32)=4.36$, $p<0.05$ and $F(1, 32)=22.90$, $p<0.0001$, respectively) such that claudin-1 expression was increased in cancer-chemotherapy animals and male animals (Fig 3B, significant posthoc in males $t_{18}=4.83$, $p<0.0001$). Tight junction protein-1 did not differ across experimental groups. In addition, intestinal expression of the pro-inflammatory cytokine tumor necrosis factor alpha (*Tnfa*), but not that of interleukin-1beta (*Il-1 β*), was increased after exposure to cancer and chemotherapy as compared to control treatment (main effect of treatment; Fig 3C; $F(1,$

32)=10.38, $p<0.005$). Lastly, *Dnmt1* expression was also increased in cancer-chemotherapy animals, (main effect of treatment; Fig 3D; $F(1, 32)=4.49$, $p<0.05$), an effect driven by male animals (significant posthoc in males $t_{18}=2.55$, $p<0.05$, females, ns). Further, *Dnmt1* expression was positively correlated with *Tnfa*, and *Cldn1* in the small intestine, but not *Il-1 β* ($r^2=0.63$, $p<.0001$, $r^2=0.63$, $p<.0001$, respectively, Fig S7). Interestingly, expression levels of *Ccl2* in PFC was significantly positively correlated with *Cldn1* expression in the small intestine, a relationship evident only in cancer-chemotherapy treated animals ($r^2=0.28$, $p<0.05$ and $r^2=0.39$, $p<0.01$, Fig 3E).

Early cancer-chemotherapy altered prefrontal cortical expression of synaptophysin long-term

To examine gene expression at a later timepoint, brains were collected from animals one week after completion of behavioral testing. At this timepoint, a significant interaction between treatment and sex was identified for *Il-1 β* expression in the PFC (Fig 4A; $F(1, 26)=5.79$, $p<0.05$) and this was due to significantly lower expression in male mice that underwent cancer-chemotherapy as compared to control males. Interestingly, cortical expression of synaptophysin, but not of *Dlg4* or gephyrin, was significantly different between groups, with a significant interaction between these factors (Fig 4B; $F(1, 28)=8.07$, $p<0.01$), and main effects for both treatment and sex ($F(1, 28)=7.85$, $p<0.01$ and $F(1, 28)=12.99$, $p<0.005$). Posthoc analyses revealed that cortical synaptophysin was significantly higher in female mice that underwent cancer-chemotherapy than in control females ($t_{16}=4.04$, $p<0.001$). There were no significant effects on the expression of white matter-related genes (*Mbp*, *Olig2*, *Pdgfra*, *Mog*), nor of *Cxcl10*, *Ccl2*, or *Tnfa* at this later timepoint.

Early cancer-chemotherapy increased adult intestinal expression of pro-inflammatory cytokines long-term

One week after completion of behavioral testing (~5 months of age), tight junction and pro-inflammatory gene expression was evaluated in the small intestine. Two-way ANOVA identified a significant interaction in the expression of *Il-1 β* (Fig 4C; $F(1, 28)=12.1$, $p<0.01$), and posthoc analysis revealed that cancer-chemotherapy exposure increased expression of IL-1 β in male animals ($t_{15}=2.98$, $p<0.01$), while expression levels did not differ in females. Similarly, two-way ANOVA identified a significant interaction in the expression of *Tnf α* (Fig 4D; $F(1, 28)=7.42$, $p<0.05$), such that cancer-chemotherapy exposure increased expression in male animals ($t_{16}=2.06$, $p<0.05$), while expression levels did not differ in females. There were no differences in expression *Cldn1* or *Tjp* between experimental groups. Further, *IL-1 β* expression in small intestine was negatively correlated with *Dlg4* and *Syp* in the PFC, only in cancer-chemotherapy treated animals (Fig 4E/F; $r^2=0.34$, $p<0.01$, $r^2=0.53$, $p<0.001$, respectively).

Correlation analysis of gene expression and behavior revealed that gephyrin in PFC was significantly positively associated with correct responses in the 5CSRTT (first session, 12 sec stimulus) and negatively correlated with premature responses (Fig 5A, B, Correct $r^2=0.33$, $p<0.01$, PREM $r^2=0.32$, $p<0.05$). Additionally, omitted responses during 5CSRTT (4 sec stimulus) were significantly positively correlated with proinflammatory gene expression in the small intestine (Fig 5C, D; *Il1 β* $r^2=0.35$, $p<0.01$, *TNF α* $r^2=0.32$, $p<0.05$).

Discussion

The primary aim of the present work was to identify potential mechanisms underlying cognitive deficits in a translationally relevant model of early life cancer and chemotherapy. Male, but not female, mice exposed early in life to cancer-chemotherapy showed recognition memory and executive function deficits. In addition, early life exposure to cancer-chemotherapy resulted acutely in increased *Ccl2* expression in the prefrontal cortex and reduced brain weight (males only) and increased *Tnfa* and claudin-1 expression in the small intestine, with decreased expression of myelin-related genes observed in both males and females. Chronically, early life exposure to cancer-chemotherapy was associated with increased intestinal expression of *Il1 β* and *Tnfa* in males and increased cortical synaptophysin expression in females. Importantly, inflammatory gene expression changes in the small intestine were correlated with adverse changes in the CNS. Collectively, these findings indicate that the present model reproduces clinically-relevant long-term survival of early-life leukemia with impairment of recognition memory and executive function in males and also identifies transient changes in expression of myelin-related genes and chemokines and lasting elevations in pro-inflammatory cytokine expression in the small intestine.

Contemporary ALL treatment protocols often combine VCR and MTX with folate rescue during the induction phase of treatment, in part based on therapeutic efficacy obtained with the L1210 mouse model of leukemia (Burchenal, 1975; Simile et al., 1993; Sirotnak et al., 1978; Skipper and Perry, 1970; Zager et al., 1973). Additionally, our model was designed to be developmentally appropriate, with the L1210 cells and chemotherapy administered in early life. This is particularly important because executive function requires the PFC (Logue and Gould, 2014), and in both rodents and humans, development of the PFC extends through adolescence (Caballero et al., 2016), which renders this area of the brain particularly vulnerable to environmental exposures during childhood and adolescence.

The five choice serial reaction time test is a translationally-relevant task to assess executive function in rodents (Bushnell and Strupp, 2009; Carli et al., 1983; Muir et al., 1992; Robbins et al., 1989). Early life cancer-chemotherapy resulted in increased premature responding in adult male mice, a response pattern that is consistent with impulsive action. Moreover, the present findings concur with studies of childhood leukemia survivors showing executive function deficits, with at least one study reporting that males were more affected than females (Cheung et al., 2016; Kanellopoulos et al., 2016; Van Der Plas et al., 2018). Further, early life cancer-chemotherapy was found to reduce recognition memory in males. Our results are consistent with similar findings in rats (Wen et al., 2018) and childhood leukemia survivors showing impaired memory, including recognition memory, years after ALL therapy (Cheung et al., 2016; Iyer et al., 2015; Phillips et al., 2020; Zhou et al., 2020). The collective 5CSRTT and NOR results indicate that, beyond survival, this is a valid model to study the long-term cognitive consequences of childhood cancer and treatment.

Importantly, early life cancer-chemotherapy exposure had no effect on motivation (measured with progressive ratio) or locomotor activity (open field environment (4 weeks later) nor in the 5CSRTT (~14 weeks later), so acute sickness behavior did not contribute to the performance deficits observed in the cognitive tasks. To allow for behavioral testing during the animals' active phase, the animals were gradually transitioned to a reverse light-dark cycle at the conclusion of chemotherapy (a 12 h shift). Animals typically require 1 day for each hour shifted, and in the present experiment, the first behavioral task (social interaction) was conducted on the 8th day, to allow for behavioral assessments as close to the conclusion of chemotherapy as possible and while animals were still young. It is possible that cancer/chemotherapy exposed animals may have differed in their adjustment to the time shift, and future experiments could be directed at understanding this question. Interestingly, the cancer/chemotherapy exposed animals performed better than controls in some tasks

(improved attentional capacity in titration and shorter time to criterion in FR1). There are other reports in the literature finding that early life immune challenges result in similar improvements in cognitive performance (Makinson et al., 2019; Zhao et al., 2021). While the explanation for improvements in these endpoints is currently unclear, it is interesting that a broad range of early life events associated with neuroimmune activity (cancer/chemotherapy, intrauterine inflammation, maternal immune activation) have certain positive effects on cognition, in addition to observed deficits.

Performance in the five choice serial reaction test in rodents depends on the integrity of the prefrontal cortex (Chudasama et al., 2003) and is modulated by local neurotransmission and neuromodulation (Carli et al., 2006; Feja and Koch, 2014; Murphy et al., 2012; Paine et al., 2015; Pezze et al., 2014; Winstanley et al., 2003; Wischhof et al., 2011). Published findings obtained in adult rodents show that chemotherapy induces neuroinflammation and epigenetic modifications (Seigers and Fardell, 2011; Wang et al., 2015; Wardill et al., 2016) and work in juvenile rodents indicates that chemotherapy can affect brain white matter (Gibson et al., 2019; Ikonomidou, 2018). Early life cancer-chemotherapy exposure was found to transiently increase PFC expression of the chemokine *Ccl2* in males and decrease expression of myelin-related genes in both males and females. Interestingly, when evaluated more than three months later, these changes were no longer present. However, at this later time point, synaptophysin expression was increased in cancer-chemotherapy-exposed females, which were overall found to be better protected against cognitive sequelae than males. This finding is also consistent with a similar study that evaluated 5CSRTT performance deficits following cisplatin chemotherapy in adult animals in which reduced performance in the task was accompanied by lower synaptophysin expression in the prefrontal cortex (Huo et al., 2018), suggesting that elevated synaptophysin expression may be protective in the females. We also found that gephyrin in PFC was positively correlated with

correct responses and negatively correlated with premature responses in cancer-chemotherapy exposed animals. Gephyrin stabilizes receptors on inhibitory synapses, and there are reports in the literature that support the involvement of PFC GABA in premature responding in the 5CSRTT, such that chronic GAD inhibition (which would result in reduced GABA levels) also increased premature responding in the 5CSRTT (Paine et al., 2015). Collectively, these results suggest that chemotherapy-associated cognitive deficits may involve dysregulation of synaptic proteins.

It has been suggested that adverse CNS consequences of cancer chemotherapy may depend on peripheral inflammatory processes (Wang et al., 2015; Wardill et al., 2016). We observed an increase in *Tnf α* expression in the small intestine of both male and female animals immediately post-chemotherapy, as well as prolonged increases in small intestine *Il-1 β* and *Tnf α* only in male animals. Acutely, expression of the DNA methylation enzyme, *Dnmt1*, was positively correlated with the expression of *Tnf α* , consistent with the finding that *Tnf α* expression in small intestine epithelial cells is regulated by DNA methylation (Marjoram et al., 2015), suggesting that alterations in DNA methylation in the small intestine may contribute to driving the proinflammatory response to early life cancer-chemotherapy exposure.

At the early time point, expression of claudin-1, essential for barrier function in the intestine (Garcia-Hernandez et al., 2017), was increased in the small intestine of cancer-chemotherapy exposed mice. An increase in *Cldn1* has also been observed in an animal model of inflammatory bowel disease (Poritz et al., 2007), a phenomenon that can be reproduced by exposure of rodent ileal intestinal epithelial cells to TNF (Poritz et al., 2011), supporting the idea that intestinal inflammation may be present in the cancer-chemotherapy animals. However histological analysis of inflammation and/or functional assays to assess intestinal permeability are important next steps to explore this possibility further. Notably, in cancer-

chemotherapy exposed animals there was a significant positive correlation between *Cldn1* expression in the small intestine and *Ccl2* expression in the PFC, a relationship that was not present in control animals. Further, *Il-1 β* expression in the small intestine was significantly negatively correlated with two synaptic proteins, PSD95 and synaptophysin. Similarly, IL-1 has been shown to lead to synaptic loss both in hippocampal slices (Sheppard et al., 2019) as well as in neuronal cultures stimulated with bacterial lipopolysaccharide (LPS) (Moraes et al., 2015). Further, we identified a significant positive correlation between expression of both *Il-1 β* and *Tnfa* in the small intestine and omitted responses, indicating a relationship between inflammatory responses in the small intestine and inattentive behavior. Collectively, these data support a relationship between inflammation in the small intestine and adverse CNS consequences in cancer-chemotherapy exposed animals.

Given the documented long-term cognitive consequences of early life chemotherapy treatment for leukemia patients, it is necessary to understand the underlying neurobiological changes that lead to such deficits. This work is best accomplished using a translationally relevant animal model that incorporates both cancer and chemotherapy at the correct developmental time. To that end, we have developed a model that results in cognitive deficits, early inflammatory gene expression changes in the prefrontal cortex and persistent inflammatory changes in the small intestine. Notably, these changes in the small intestine were correlated with a number of adverse CNS outcomes, including proinflammatory and synaptic gene expression changes, as well as executive function deficits. Based on these observations, we hypothesize that a gut-to-brain pathway may be an essential component underlying cognitive deficits associated with chemotherapy. Whether strategies to limit small intestine inflammation/damage may improve cognitive outcomes in leukemia survivors will be an important future question to address.

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Supplemental methods

Social interaction. Social interaction was assessed at six weeks of age corresponding to three weeks after the start of chemotherapy, upon introduction of a conspecific mouse into the home cage of the experimental mice. Behavior was videotaped for 5 minutes and scored by a rater blind to the experimental condition. The following parameters were scored: time spent in approach, time spent in direct contact, mean approach duration, and mean contact duration.

Open field. At 9-10 weeks of age or 4-5 weeks after the start of chemotherapy, mice were introduced, one hour after lights-off, into the center of an empty test chamber (L: 46 x W: 46 x H: 40.6 cm) and allowed to freely explore for 5 min under red light illumination. The next day, the animal was again placed into the center of an empty test chamber, but this time under bright white light conditions, and allowed to explore for 5 min. Both sessions were recorded and analyzed for locomotor activity using TopScan (CleverSys) automated tracking software. After each trial the chamber was cleaned with 70% ethanol solution and water.

Novel object recognition. The day after the open field session under bright light mice were again placed in the center of the test chamber, which now contained two identical objects placed 20 cm apart and were allowed to investigate for 5 min. Twenty-four hours later mice were put one more time into the test chamber, which now contained a familiar object from the previous session and one novel object, and allowed to freely explore for 5 min. Both sessions were recorded and investigation time of the objects was measured using BORIS© behavioral event logging software. Object investigation time was defined as any observable sniffing or nose proximity (within 2 cm) to an object. After each trial the chamber and objects were cleaned with 70% ethanol solution and water.

Operant behavior. At 11 weeks of age or 8 weeks after the start of chemotherapy, animals were food-restricted to 85-90% of their free feeding body weight over the course of a week and animals were pre-exposed to the reinforcer (Yoo-hoo, Dr. Pepper Snapple Group Inc., Plano, TX, USA) for 24 hours in the home cage. Operant testing was carried out in Bussey-Saksida Mouse Touch Screen operant chambers (Lafayette Instrument, Lafayette, IN, USA) running ABET II Touch software as previously described (Makinson et al., 2019; Smith et al., 2020). Training (30 minute sessions) occurred five days a week between 1000 and 1400, during the dark phase. The back of the chamber contains the touchscreen and the front of the chamber contains a magazine, where reinforcement is delivered. Infrared beam breaks upon magazine entry were recorded. Prior to cognitive testing, mice were given a 3 day habituation training protocol to familiarize with test chamber and reward. Each habituation session began with an illuminated magazine and a 5 sec feeder pulse (all future trials were 280 ms pulses). The magazine light remained on until the mouse collected the reward. Once the mouse collected the reward, there was a 10 sec inter trial interval (ITI). Touch-pads were inactive for entire habituation session.

Fixed ratio 1. Following magazine training, Fixed Ratio 1 (FR1) training began. The center stimulus was illuminated until one touch was made to it. The light was then terminated and reinforcer was dispensed into the magazine. When the animal completed a magazine entry, the cycle repeated. Criterion for task acquisition was 70 responses within the 30 minute session. Animals that failed to reach criterion were eliminated from FR1 and subsequent progressive ratio (PR) analyses.

Progressive ratio. Progressive ratio testing occurred after animals reached criterion in FR1. The number of responses to dispense reinforcer now increased arithmetically every third trial

(i.e. 1, 1, 1, 2, 2, 2, 4, 4, 4, 7, 7, 7...). Sessions continued for 60 minutes or until the animal made no response for 5 minutes. Breakpoint was measured at the trial in which the animal would no longer respond for the reinforcer.

Five choice serial reaction time task. Animals that met FR1 criterion continued onto 5 choice serial reaction (5CSRTT) training, as previously described (Grissom et al., 2014; Smith et al., 2020). Briefly, animals initiated the training by completion of a magazine entry. This triggered an inter-trial interval (ITI) through which the animal waited for the illumination of one of the five stimulus areas at the back of the chamber. The animal then was required to touch the illuminated area for completion of a correct trial. A premature trial was recorded if an animal made a response during the ITI. If an animal responded to an un-illuminated area then an incorrect trial was recorded. If no response was made, an omitted trial was recorded. Only a response at the correct hole during the 8-second stimulus or a 2-second limited hold after stimulus termination was reinforced. Animals moved through 3 progressively more difficult 5CSRTT schedules, as defined by decreasing stimulus length (Session 10: 16 seconds, Session 11: 8 seconds, Session 12: 4 seconds). Performance measures are reported for first day where criterion (>20 responses, with 50% correct) was met.

Five choice serial reaction titration schedule. After the 5CSRTT, the animals moved on to the titration schedule, a more challenging version of the task, as described in Martin et al. (2015). Briefly, in contrast to the standard 5CSRTT in which the stimulus duration and ITI remained constant throughout the session, within a single session of the titration schedule, stimulus duration is titrated based on an animals' individual performance. Specifically, after a successful magazine entry to initiate training for the session, the first light stimulus is 10 seconds (stimulus duration). Upon each correct response, reinforcer is received, and the

stimulus duration for the subsequent trial is shortened one step down in the series (10, 8, 6, 4, 2, 1, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 seconds respectively). Incorrect and omitted responses elicit a lengthened stimulus duration one step up the series for the subsequent trial. While premature responses maintain the same stimulus duration. The ITI remained constant for this training. Median stimulus duration (median of stimulus lengths for all trials) and shortest cue duration (shortest stimulus duration reached within a single training session) were calculated for each animal. Performance measures are reported for day 1 of titration training.

Immunohistochemical localization of Iba1. Fixed brains were removed and placed for 4 hours in the same fixative and then in 30% sucrose for 24 hours at 4°C. Thirty- μ m brain sections were cut on a sliding microtome and stored in a RNase-free cryoprotective solution (20 % glycerol, 30 % ethylene glycol in PBS, 0.1 M, pH 7.4) at -20 °C. Brain slices were rinsed in KPBS then 3% hydrogen peroxide and rinsed again in KPBS followed by 1% sodium borohydrate. Tissue was incubated overnight at 4°C in primary antibody (1:2000 IBA1, Wako, 019-19741). Tissue was rinsed again in KPBS before being incubated in biotinylated goat antirabbit secondary antibody (Vector Laboratories, Burlingame, CA, BA-1000) for 1 hour, then washed and incubated in avidin-biotin solution (Vector laboratories, PK-6100) for 1 hour. Immunoreactivity was visualized with 3,3'-diaminobenzine. After mounting, slides were dehydrated with graded ethanol and coverslipped with DPX. Images were collected at 10x for counts and 40x for perimeter and area analysis. For counts, templates were created in Photoshop (Adobe Photoshop, Adobe Systems, San Jose, CA, USA) and overlaid on each image to ensure equal tissue area was included in each count. All cell counting was performed manually using the cell counter function of ImageJ (<http://imagej.nih.gov/ij/>). For perimeter and area measurements, the perimeter of the soma of each cell was traced manually using the Freehand Selections tool on ImageJ, then measured automatically.

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Figure legends

Figure 1. Cancer-chemotherapy affects social approach, recognition memory and performance in the 5CSRTT. (A) Mean approach duration during the social interaction task was significantly lower in animals exposed to cancer-chemotherapy, as well as in males as compared to females. (B) All animals were able to distinguish the novel object (discrimination index significantly different than 0.5), however, male cancer-chemotherapy exposed animals had a discrimination index significantly lower than controls. (C) Male cancer-chemotherapy exposed mice showed an increase in premature responding in the 5CSRTT, while females did not differ. (controls: white circle, cancer-chemotherapy: black triangle). * $p < 0.05$, ** $p < 0.01$

Figure 2. Cancer-chemotherapy affects brain weight and white matter. (A) Cancer-chemotherapy significantly reduced overall brain weight in male, but not female animals. (B, C) Cancer-chemotherapy reduced *Mbp* and *Olig2* mRNA expression in PFC of both male and female animals. (controls: white circle, cancer-chemotherapy: black triangle). * $p < 0.05$, ** $p < 0.01$

Figure 3. Cancer-chemotherapy acutely alters gene expression in PFC and small intestine. (A) *Ccl2* mRNA expression was increased in the PFC of male animals exposed to cancer-chemotherapy. (B, C) Cancer-chemotherapy led to an increase in (B) claudin1 and (C) *Tnf α* mRNA expression in small intestine in both male and female animals and (D) *Dnmt1* mRNA expression in small intestine was increased in male animals exposed to cancer-chemotherapy. (E). Expression of *Ccl2* in PFC and *Cldn1* in small intestine were positively

correlated in cancer-chemotherapy exposed mice. # main effect of treatment, $p < 0.05$, *significant posthoc difference, $p < 0.05$. (controls: white circle (dotted line), cancer-chemotherapy: black triangle (solid line)).

Figure 4. Cancer-chemotherapy alters gene expression in PFC and small intestine long term. *Il1 β* gene expression in PFC (A) was decreased in male animals exposed to cancer-chemotherapy, while synaptophysin expression (B) was significantly increased in female mice exposed to cancer-chemotherapy. In the small intestine, *Il1 β* (C) and *Tnfa* (D) mRNA was significantly increased in male animals exposed to cancer-chemotherapy. IL-1 β expression in small intestine was negatively correlated with *Dlg4* (E) and *Syp* (F) in the PFC, only in cancer-chemotherapy treated animals. * $p < 0.05$, *** $p < 0.005$, **** $p < 0.0001$ (controls: white circle/dotted line, cancer-chemotherapy: black triangle/solid line).

Figure 5. Correlation of gene expression and performance in 5CSRTT. Gephyrin in PFC positively correlates with correct responses (A) and negatively with premature responses (B) in the 5CSRTT, only in animals exposed to cancer-chemotherapy. Omissions in the 5CSRTT were positively correlated with *Il1 β* expression (C) and *Tnfa* (D) expression in the small intestine only in animals exposed to cancer-chemotherapy. (controls: white circle/dotted line, cancer-chemotherapy: black triangle/solid line).

Figure 1

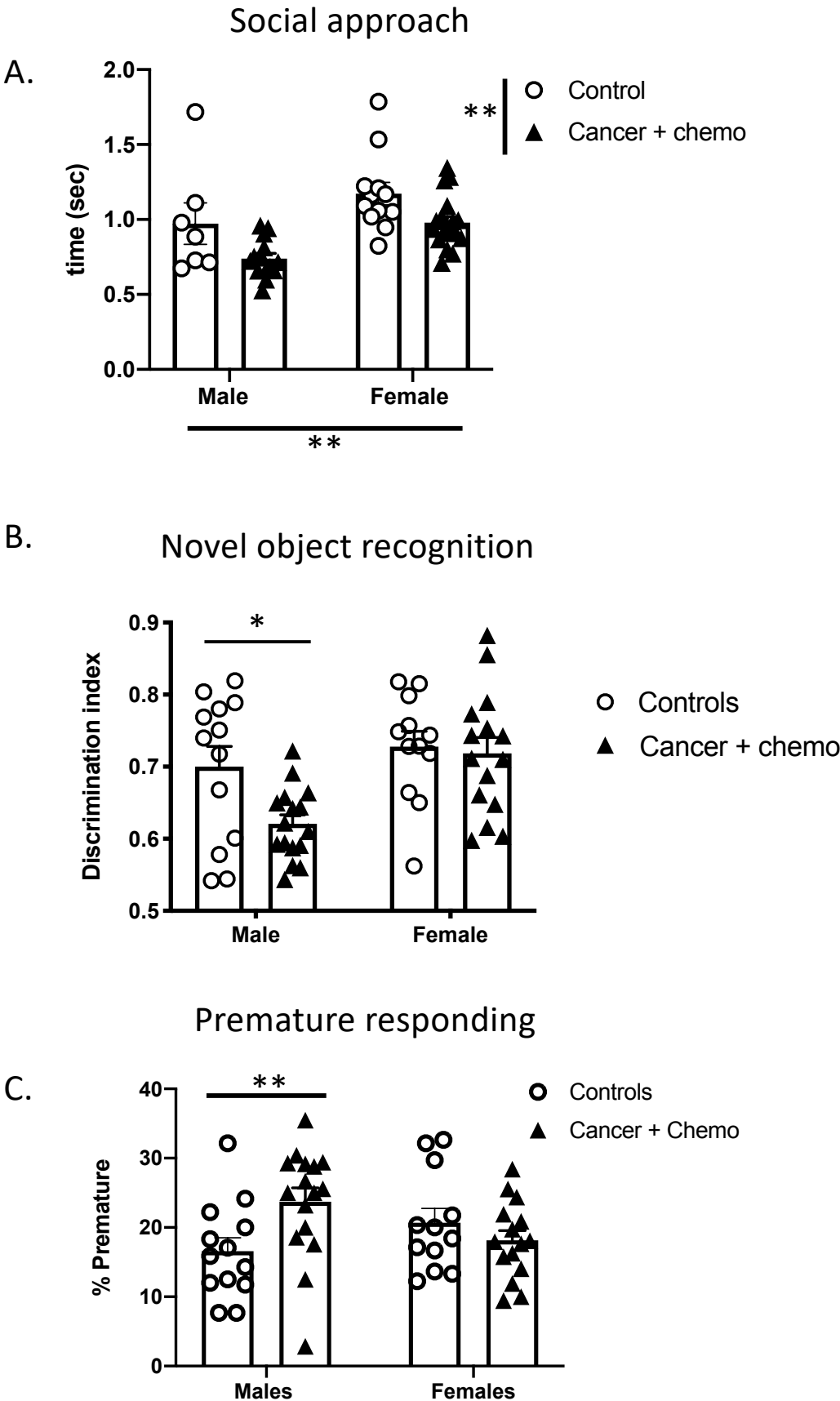


Figure 2

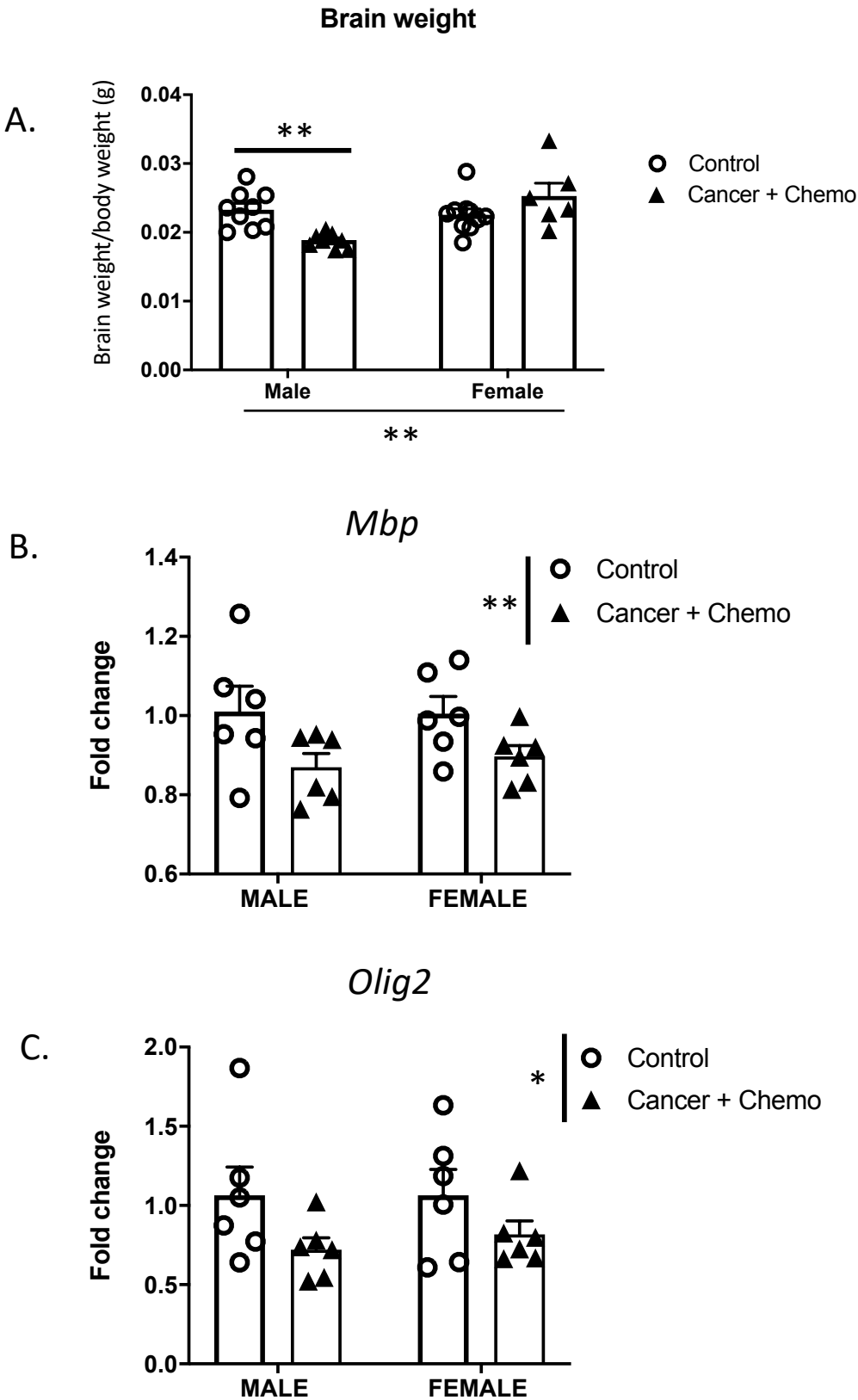


Figure 3

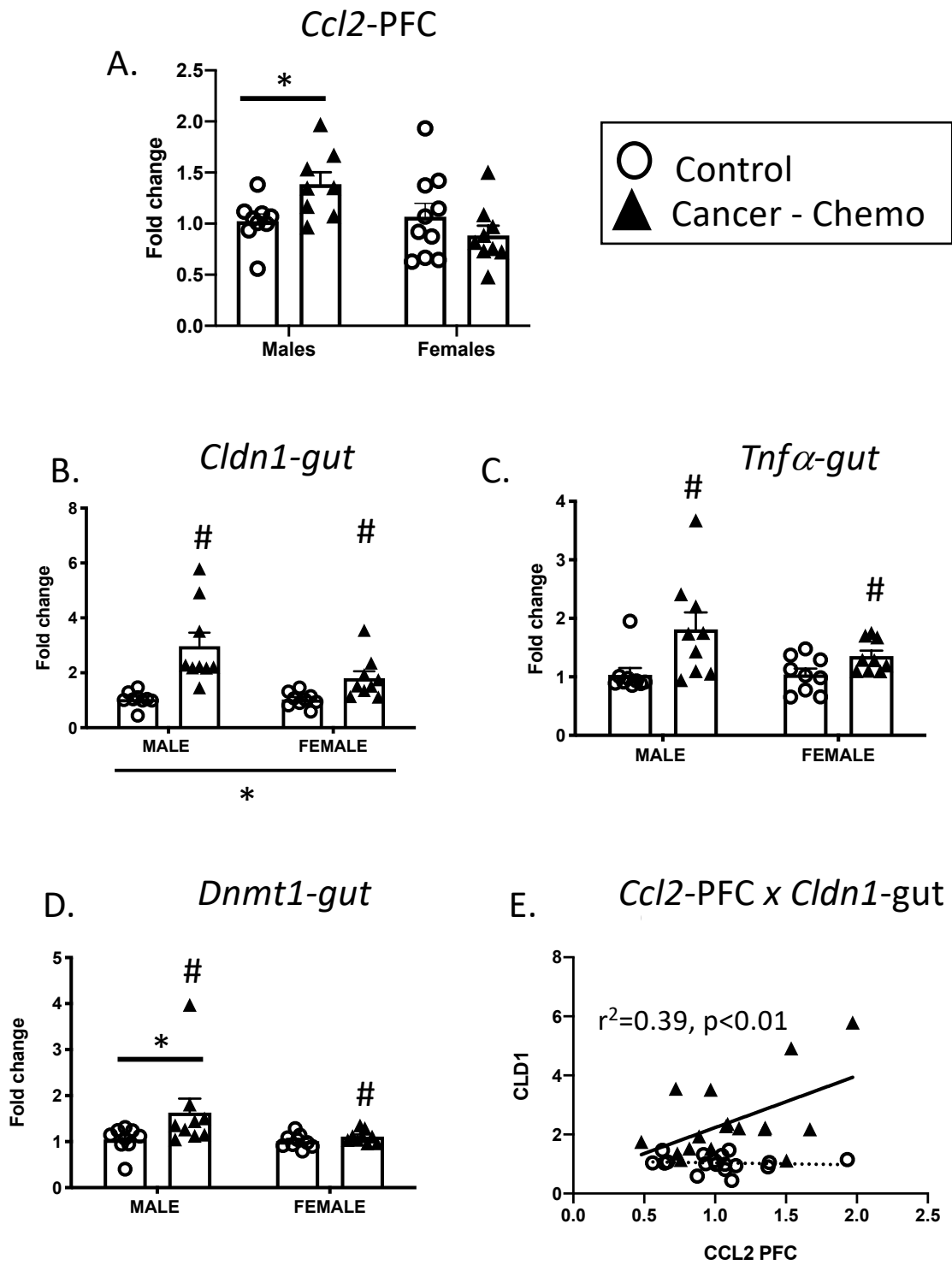


Figure 4

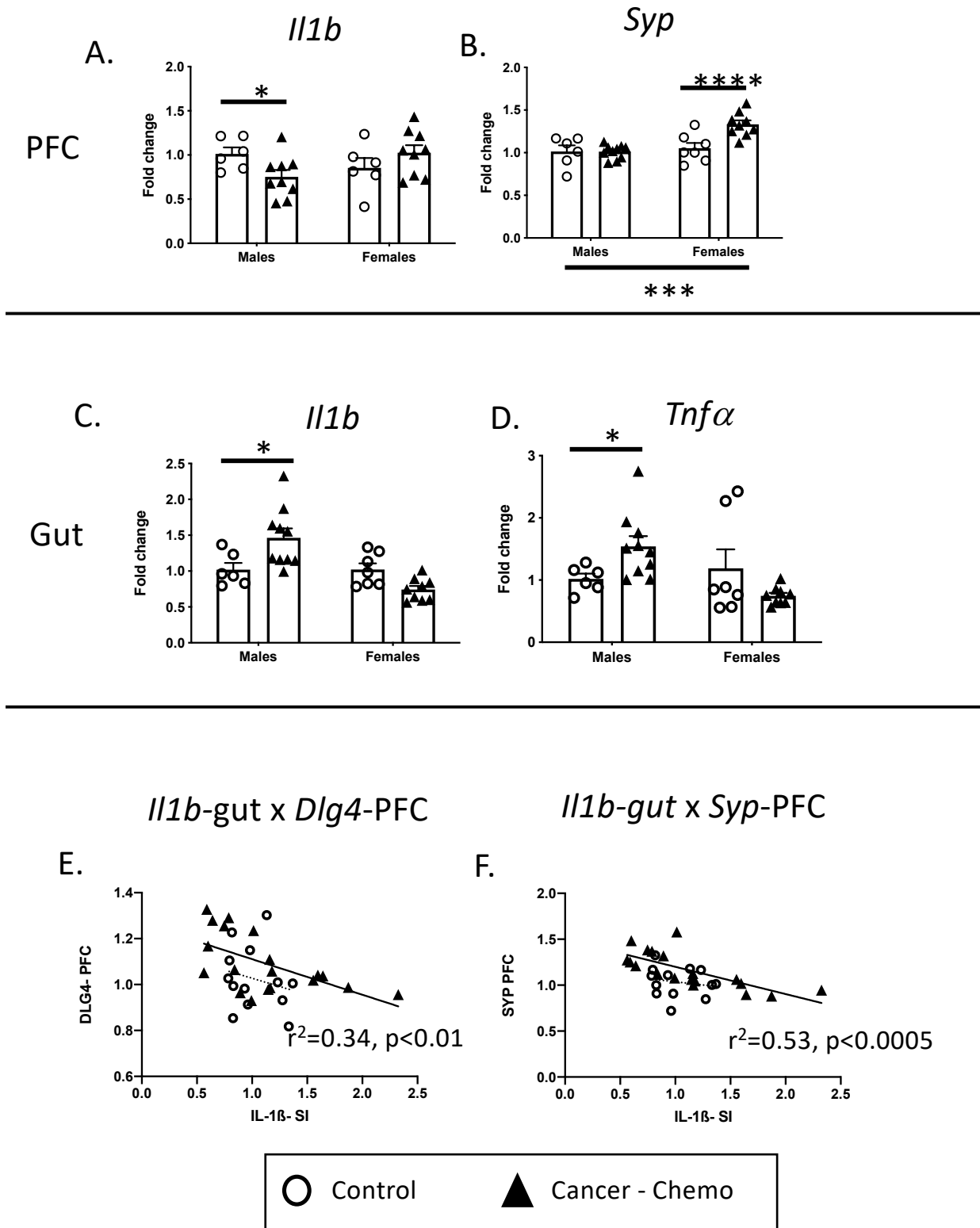
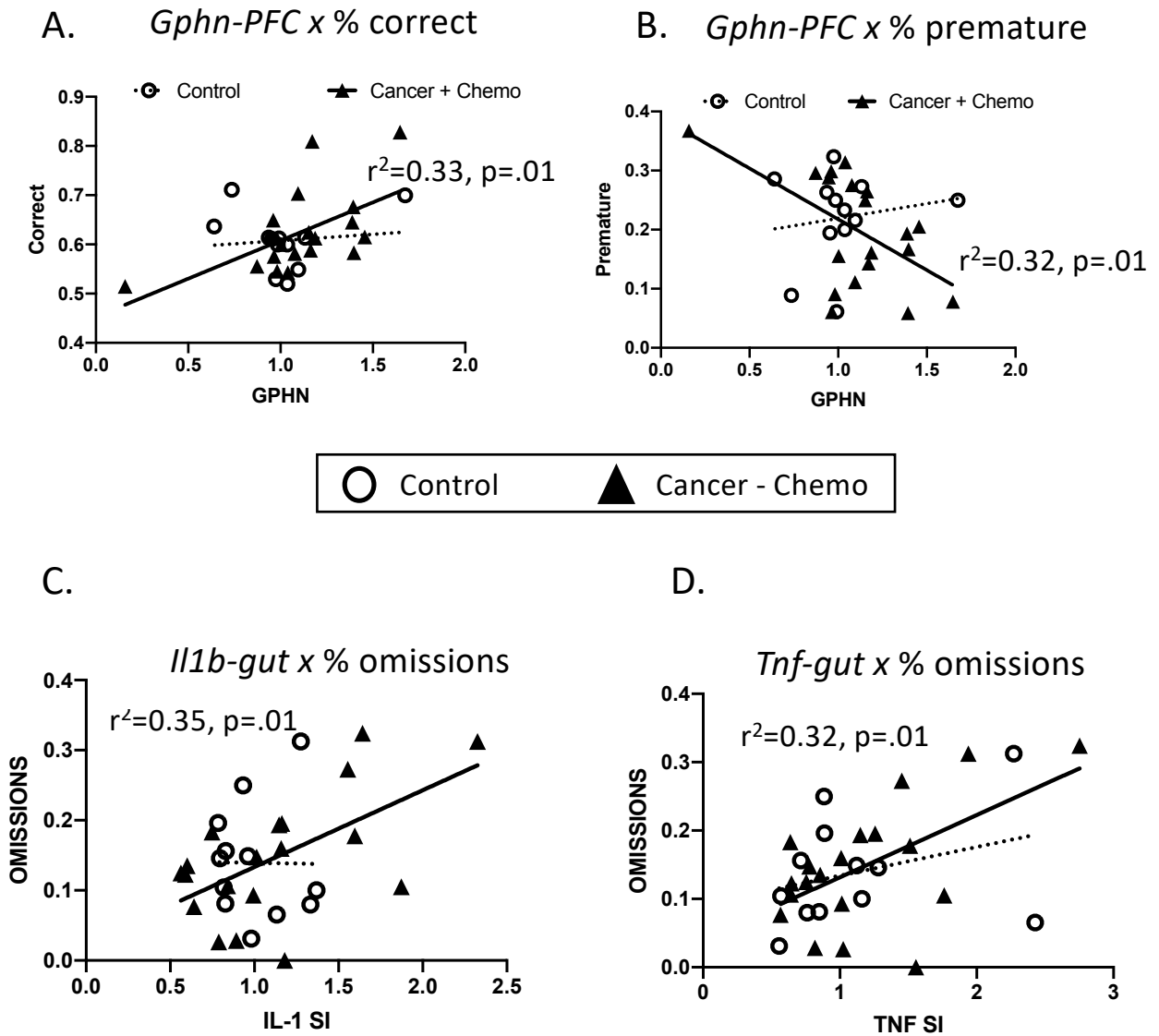


Figure 5



Supplemental methods

Social interaction. Social interaction was assessed at six weeks of age corresponding to three weeks after the start of chemotherapy, upon introduction of a conspecific mouse into the home cage of the experimental mice. Behavior was videotaped for 5 minutes and scored by a rater blind to the experimental condition. The following parameters were scored: time spent in approach, time spent in direct contact, mean approach duration, and mean contact duration.

Open field. At 9-10 weeks of age or 4-5 weeks after the start of chemotherapy, mice were introduced, one hour after lights-off, into the center of an empty test chamber (L: 46 x W: 46 x H: 40.6 cm) and allowed to freely explore for 5 min under red light illumination. The next day, the animal was again placed into the center of an empty test chamber, but this time under bright white light conditions, and allowed to explore for 5 min. Both sessions were recorded and analyzed for locomotor activity using TopScan (CleverSys) automated tracking software. After each trial the chamber was cleaned with 70% ethanol solution and water.

Novel object recognition. The day after the open field session under bright light mice were again placed in the center of the test chamber, which now contained two identical objects placed 20 cm apart and were allowed to investigate for 5 min. Twenty-four hours later mice were put one more time into the test chamber, which now contained a familiar object from the previous session and one novel object, and allowed to freely explore for 5 min. Both sessions were recorded and investigation time of the objects was measured using BORIS© behavioral event logging software. Object investigation time was defined as any observable sniffing or nose proximity (within 2 cm)

to an object. After each trial the chamber and objects were cleaned with 70% ethanol solution and water.

Operant behavior. At 11 weeks of age or 8 weeks after the start of chemotherapy, animals were food-restricted to 85-90% of their free feeding body weight over the course of a week and animals were pre-exposed to the reinforcer (Yoo-hoo, Dr. Pepper Snapple Group Inc., Plano, TX, USA) for 24 hours in the home cage. Operant testing was carried out in Bussey-Saksida Mouse Touch Screen operant chambers (Lafayette Instrument, Lafayette, IN, USA) running ABET II Touch software as previously described {Makinson, 2019 #7024;Smith, 2020 #7023}. Training (30 minute sessions) occurred five days a week between 1000 and 1400, during the dark phase. The back of the chamber contains the touchscreen and the front of the chamber contains a magazine, where reinforcement is delivered. Infrared beam breaks upon magazine entry were recorded. Prior to cognitive testing, mice were given a 3 day habituation training protocol to familiarize with test chamber and reward. Each habituation session began with an illuminated magazine and a 5 sec feeder pulse (all future trials were 280 ms pulses). The magazine light remained on until the mouse collected the reward. Once the mouse collected the reward, there was a 10 sec inter trial interval (ITI). Touch-pads were inactive for entire habituation session.

Fixed ratio 1. Following magazine training, Fixed Ratio 1 (FR1) training began. The center stimulus was illuminated until one touch was made to it. The light was then terminated and reinforcer was dispensed into the magazine. When the animal completed a magazine entry, the cycle repeated. Criterion for task acquisition was 70 responses within the 30 minute session. Animals that failed to reach criterion were eliminated from FR1 and subsequent progressive ratio (PR) analyses.

Progressive ratio. Progressive ratio testing occurred after animals reached criterion in FR1. The number of responses to dispense reinforcer now increased arithmetically every third trial (i.e. 1, 1, 1, 2, 2, 2, 4, 4, 4, 7, 7, 7...). Sessions continued for 60 minutes or until the animal made no response for 5 minutes. Breakpoint was measured at the trial in which the animal would no longer respond for the reinforcer.

Five choice serial reaction time task. Animals that met FR1 criterion continued onto 5 choice serial reaction (5CSRRT) training, as previously described {Grissom, 2014 #7025; Smith, 2020 #7023}. Briefly, animals initiated the training by completion of a magazine entry. This triggered an inter-trial interval (ITI) through which the animal waited for the illumination of one of the five stimulus areas at the back of the chamber. The animal then was required to touch the illuminated area for completion of a correct trial. A premature trial was recorded if an animal made a response during the ITI. If an animal responded to an un-illuminated area then an incorrect trial was recorded. If no response was made, an omitted trial was recorded. Only a response at the correct hole during the 8-second stimulus or a 2-second limited hold after stimulus termination was reinforced. Animals moved through 3 progressively more difficult 5CSRRT schedules, as defined by decreasing stimulus length (Session 10: 16 seconds, Session 11: 8 seconds, Session 12: 4 seconds). Performance measures are reported for first day where criterion (>20 responses, with 50% correct) was met.

Five choice serial reaction titration schedule. After the 5CSRRT, the animals moved on to the titration schedule, a more challenging version of the task, as described in Martin et al. {, 2015

#7026}. Briefly, in contrast to the standard 5CSRTT in which the stimulus duration and ITI remained constant throughout the session, within a single session of the titration schedule, stimulus duration is titrated based on an animals' individual performance. Specifically, after a successful magazine entry to initiate training for the session, the first light stimulus is 10 seconds (stimulus duration). Upon each correct response, reinforcer is received, and the stimulus duration for the subsequent trial is shortened one step down in the series (10, 8, 6, 4, 2, 1, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 seconds respectively). Incorrect and omitted responses elicit a lengthened stimulus duration one step up the series for the subsequent trial. While premature responses maintain the same stimulus duration. The ITI remained constant for this training. Median stimulus duration (median of stimulus lengths for all trials) and shortest cue duration (shortest stimulus duration reached within a single training session) were calculated for each animal. Performance measures are reported for day 1 of titration training.

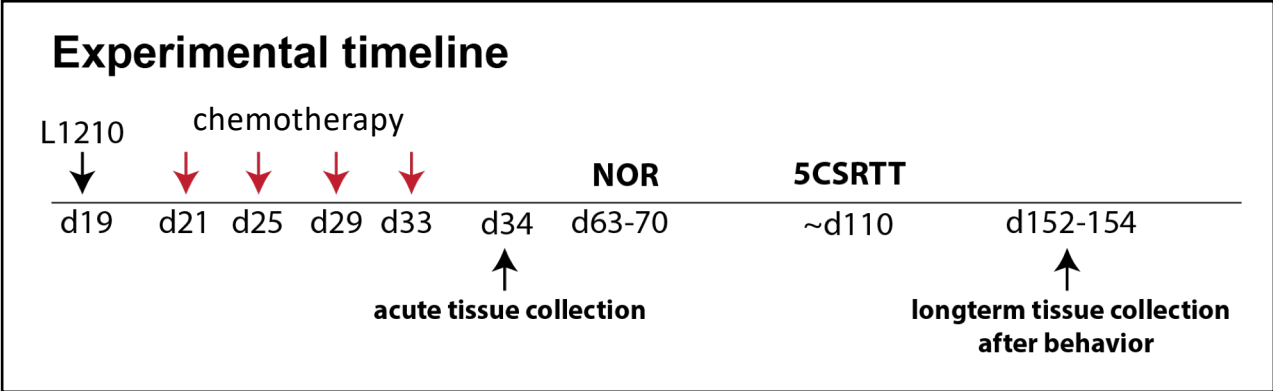
Immunohistochemical localization of Iba1. Fixed brains were removed and placed for 4 hours in the same fixative and then in 30% sucrose for 24 hours at 4°C. Thirty- μ m brain sections were cut on a sliding microtome and stored in a RNase-free cryoprotective solution (20 % glycerol, 30 % ethylene glycol in PBS, 0.1 M, pH 7.4) at -20 °C. Brain slices were rinsed in KPBS then 3% hydrogen peroxide and rinsed again in KPBS followed by 1% sodium borohydrate. Tissue was incubated overnight at 4°C in primary antibody (1:2000 IBA1, Wako, 019-19741). Tissue was rinsed again in KPBS before being incubated in biotinylated goat antirabbit secondary antibody (Vector Laboratories, Burlingame, CA, BA-1000) for 1 hour, then washed and incubated in avidin-biotin solution (Vector laboratories, PK-6100) for 1 hour. Immunoreactivity was visualized with 3,3'-diaminobenzidine. After mounting, slides were dehydrated with graded ethanol and

coverslipped with DPX. Images were collected at 10x for counts and 40x for perimeter and area analysis. For counts, templates were created in Photoshop (Adobe Photoshop, Adobe Systems, San Jose, CA, USA) and overlaid on each image to ensure equal tissue area was included in each count. All cell counting was performed manually using the cell counter function of ImageJ (<http://imagej.nih.gov/ij/>). For perimeter and area measurements, the perimeter of the soma of each cell was traced manually using the Freehand Selections tool on ImageJ, then measured automatically.

Table S1

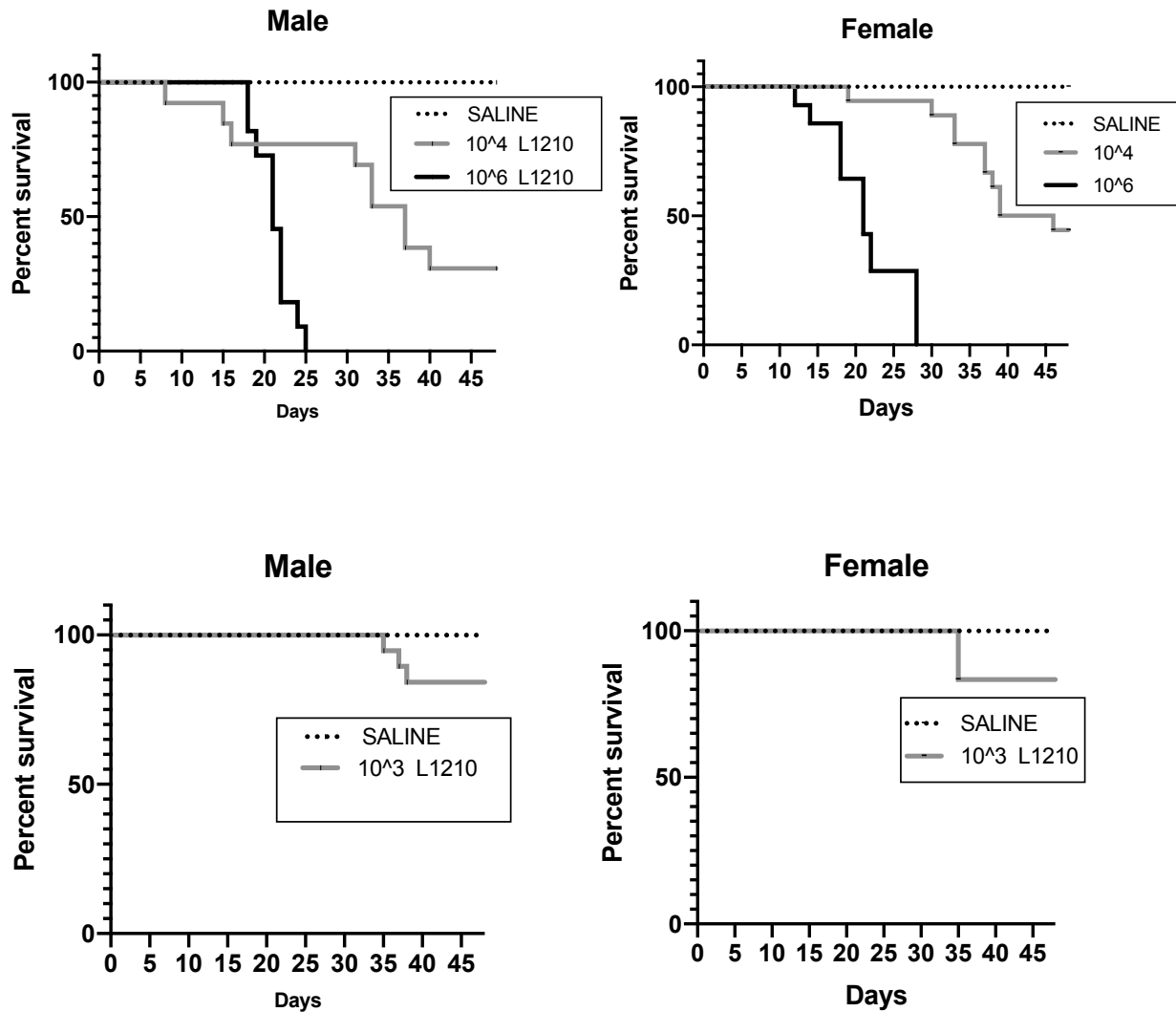
Gene name	Common name	Assay ID
Actb	actin, beta	Mm00607939_s1
Ccl2	c-c motif chemokine ligand 2	Mm00441242_m1
Cldn1	claudin 1	Mm00516701_m1
Cxcl10	C-X-C motif chemokine ligand 10	Mm00445235_m1
Dlg4	post synaptic density protein 95, PSD95	Mm00492193_m1
Dnmt1	DNA methyltransferase 1	Mm00599763_m1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Mm00484668_m1
Gphn	gephyrin	Mm00556895_m1
Il1b	interleukin 1 beta	Mm00434228_m1
Itgam	cd11b, complement receptor 3	Mm01271250_m1
Mbp	myelin basic protein	Mm01266402_m1
Mog	myelin oligodendrocyte glycoprotein	Mm00447824_m1
Olig2	oligodendrocyte Transcription Factor 2	Mm01210556_m1
Pdgfr-alpha	platelet derived growth factor receptor alpha	Mm00440701_m1
Syp	synaptophysin	Mm00436850_m1
Tjp1	tight junction protein 1	Mm01320638_m1
Tnf-alpha	tumor necrosis factor alpha	Mm00443258_m1

Supplemental Figure 1



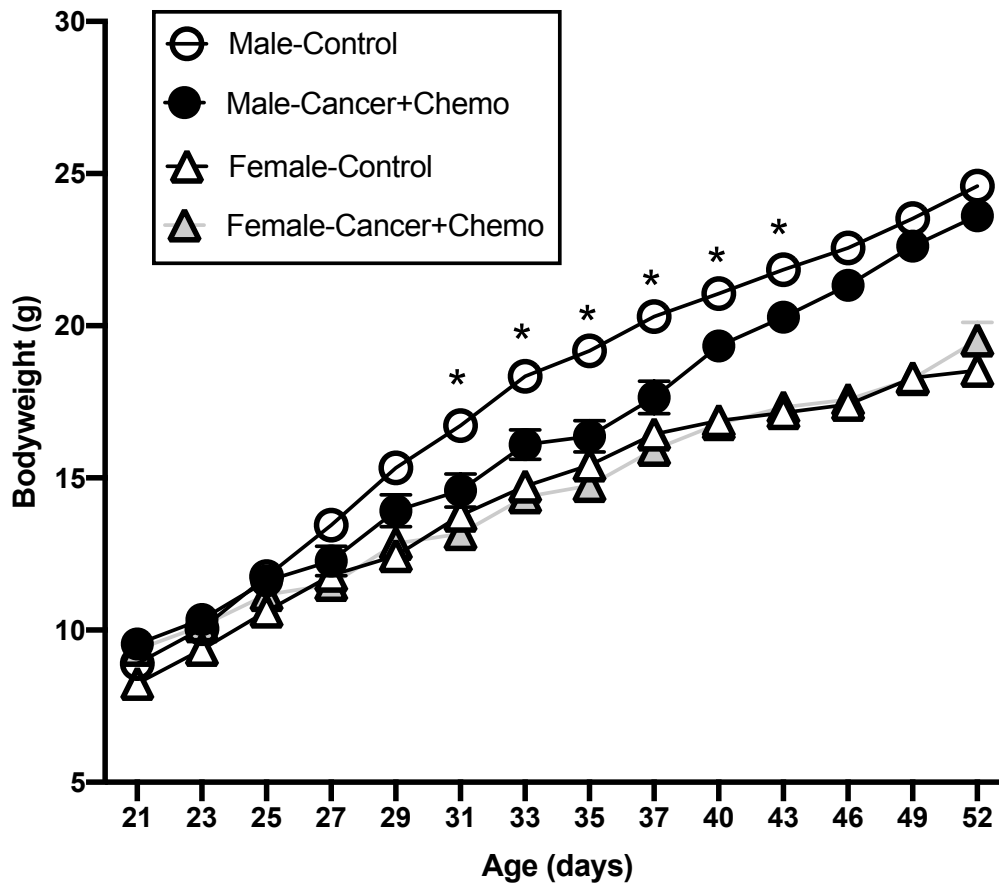
Experimental timeline for cancer and chemotherapy administration, with timing of behavior and tissue collection indicated.

Supplemental Figure 2



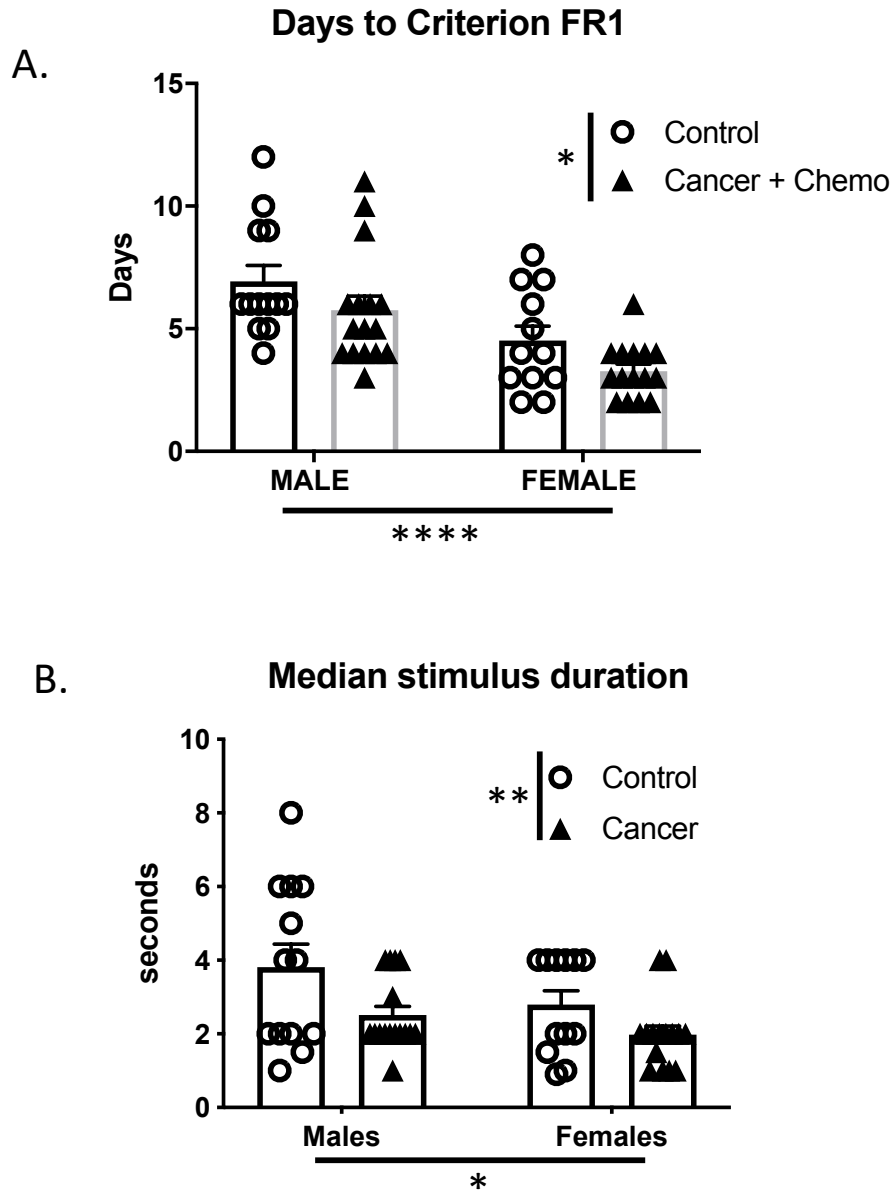
Survival curves. Top row shows male (left) and female (right) survival curves comparing saline (dotted line), 10^4 L1210 cells (gray line) and 10^6 cells (black line). Male (n=11, 13, 14, respectively) and female (n=11, 18, 14, respectively). Bottom row shows male (left) and female (right) survival curves comparing saline (dotted line) and 10^3 L1210 cells (gray line). Male (n= 13, 19, respectively) and female (n=12, 15, respectively).

Supplemental Figure 3



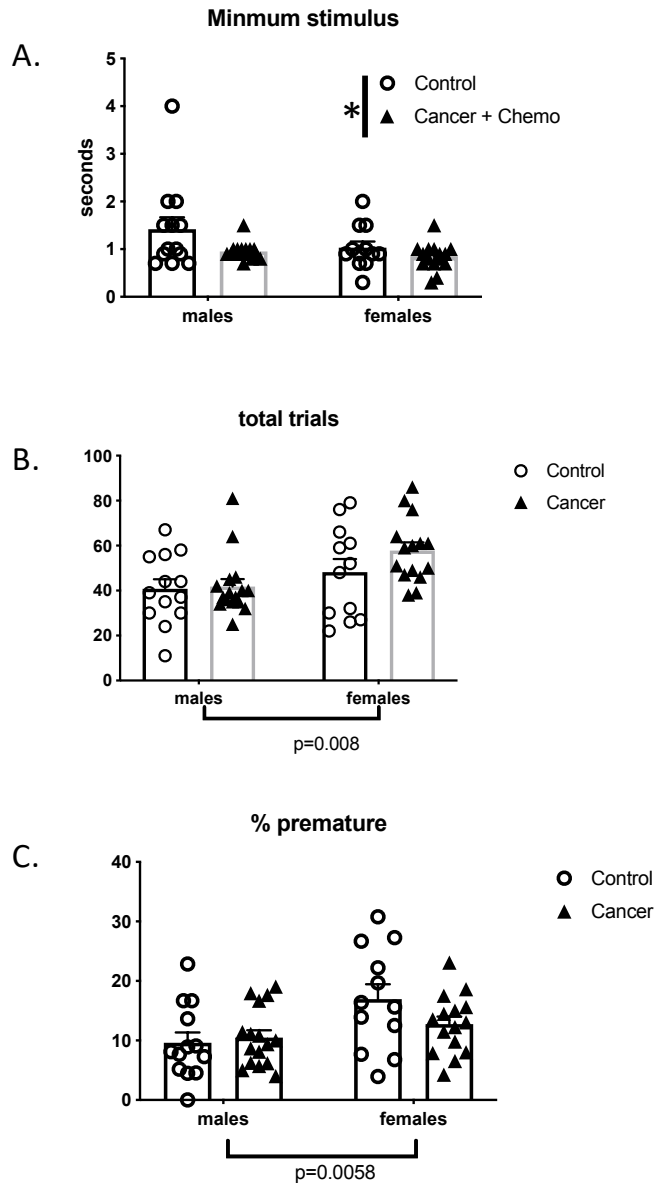
Body weight (g) from P21 to P52. Bodyweight of male cancer/chemotherapy exposed animals was significantly lower than saline injected animals from p33-p43. Cancer/chemotherapy exposure did not lead to a group differences in female mice. * $p < 0.05$ (male cancer/chemotherapy vs male control). Male control (white circle; $n=13$), male cancer/chemotherapy (black circle, $n=19$), female control (white triangle; $n=12$), female cancer/chemotherapy (gray triangle, $n=18$).

Supplemental Figure 4



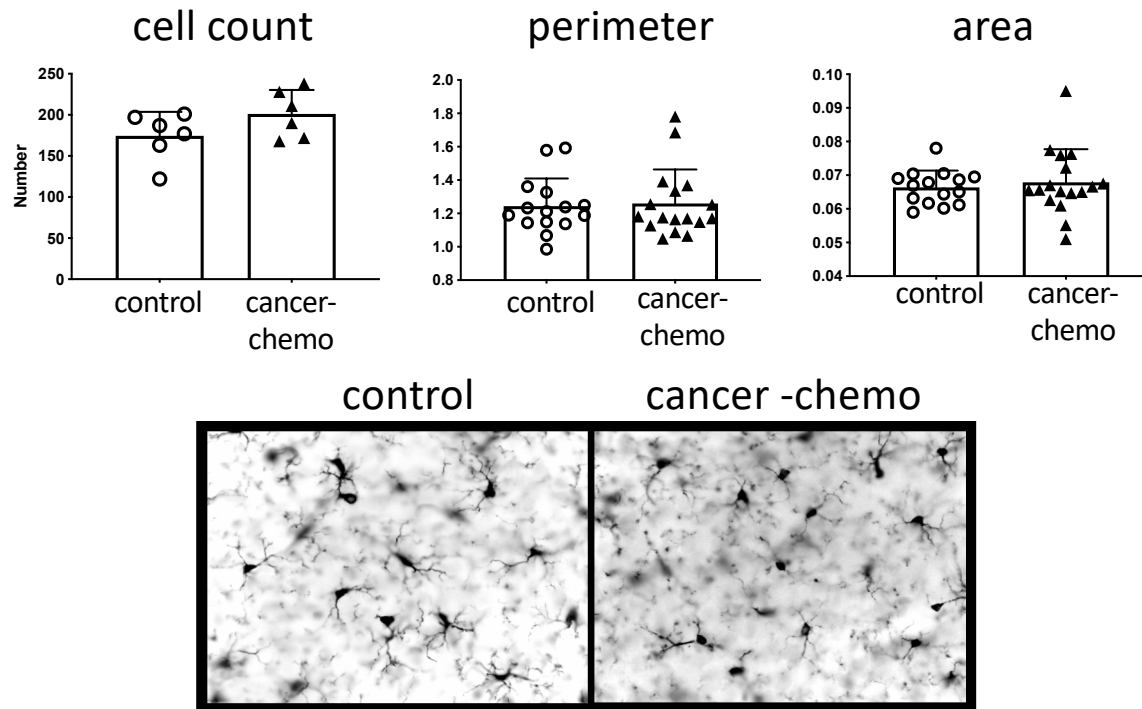
Cancer-chemotherapy affects performance in 5CSRTT. (A) Cancer-chemotherapy exposed animals and females reached criterion more rapidly than controls or males, respectively. (B) Cancer-chemotherapy exposed animals and females reached a shorter stimulus length during titration as compared to controls or males, respectively. (controls- white circle, cancer-chemotherapy- black triangle). * $p < 0.05$, ** $p < 0.01$

Supplemental Figure 5



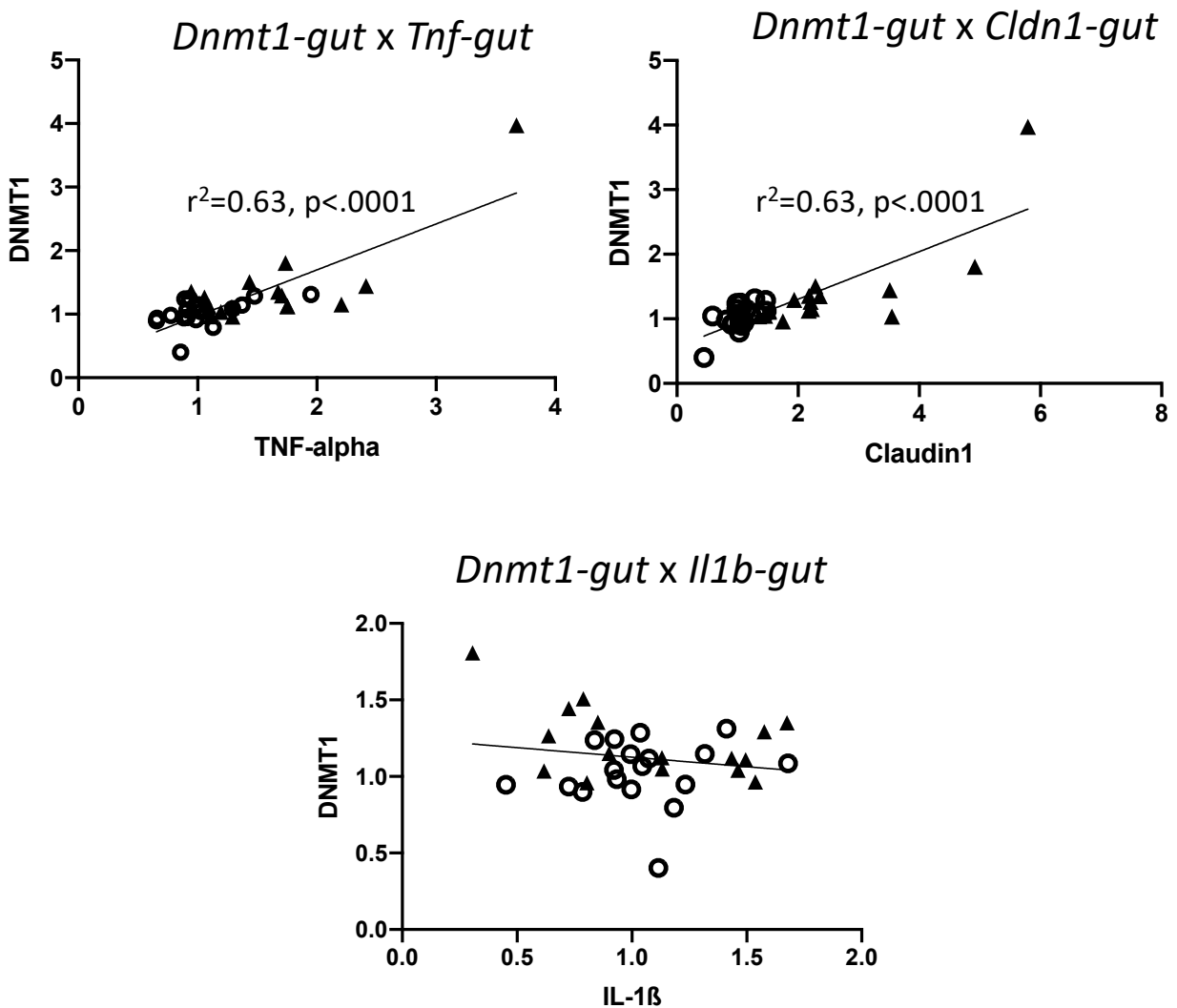
Cancer-chemotherapy affects performance in 5CSRRT. (A) Cancer+chemotherapy exposed animals reached a shorter minimum stimulus length during titration as compared to controls. (B) During titration, female mice completed more trials (B) and had more premature responses (C) than male animals. (controls- white circle, cancer+chemotherapy- black triangle). * $p < 0.05$

Supplemental Figure 6



Microglial analysis in prefrontal cortex . Cancer +chemotherapy exposure did not affect microglial number, microglial size (perimeter) or microglial area. Representative photomicrographs of Iba1 stained tissue.

Supplemental Figure 7



Correlation of DNMT1 with proinflammatory molecules in the small intestine.

DNMT1 expression in small intestine was positively correlated with TNF- α (A) and claudin1 (B) in the small intestine acutely, but not with IL-1 β (C). (controls- white circle, cancer+chemotherapy- black triangle).