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Mammary tumors suppress aging-induced neuroinflammation in female Balb/c mice

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ABSTRACT

Neuroinflammation confers changes in brain function (i.e., behavior) that are hypothesized to be adaptive in the short-term, but detrimental (e.g., depression, anxiety) if they persist. Both peripheral tumor growth (outside of the brain) and natural aging independently cause neuroinflammation in rodents, which is corroborated by clinical studies. Mammary tumor effects on neuroinflammation and behavior, however, are typically studied in young rodents, whereas most breast cancer patients are middle-aged. Therefore, the existing literature likely underestimates the resulting neuroinflammation that may occur in clinical cancer populations. The present study tested the hypothesis that aging exacerbates mammary tumor-induced neuroinflammation in female mice. Aging (16 months and ovariectomized) increased body and spleen masses, whereas tumors grew faster and increased spleen mass in young mice (12 weeks) only. Tumors (IL-6, IL-10, TNFα, MCP-1, CXCL1, IP-10) and aging (IL-10, IFNγ) independently increased circulating inflammatory markers, although these variables were only significantly additive in one case (TNFα). In contrast to our prediction, the interaction between tumors and aging resulted in reduced mRNA and protein expression of select inflammatory markers in the hippocampus of tumor-bearing aged mice relative to aged controls. These results indicate that tumors reduce inflammatory activation in the brains of aged mice, a deficit that is likely disadvantageous. Further understanding of how aging and cancer interact to affect brain function is necessary to provide clinically-relevant results and identify mechanisms underlying persistent behavioral issues hampering adult cancer patients.

1. Introduction

In humans, advanced age is associated with heightened neuroinflammation following peripheral inflammatory stimuli [1,2] and causes precipitous impairments in the functions mediated by the affected brain regions [3–5]. In rats, the normal aged brain exists in a subtle, but primed neuroinflammatory state [6], causing exacerbated inflammation only in response to various immune, stressor, and metabolic challenges than compared to the young adult brain [6,7].

In young adult tumor-bearing rodents, select proinflammatory cytokines are modestly increased in some areas of the resting brain (e.g., hippocampus [8,9]) and neuroinflammatory pathways are sensitized to subsequent challenges, similar to aging models [10]. However, current rodent cancer models consist virtually exclusively of healthy young adults, whereas, the human cancer populations they were designed to model are largely middle aged (45–65 years of age) and menopausal. For example, breast cancer incidence is approximately seven times higher in women ages 50+ years than in women between the ages of 20–49 [11].

Thus, middle-aged women are especially vulnerable to neuroinflammatory insults. The oversight of advanced age in cancer modeling likely oversimplifies and underestimates the impact that natural physiological aging contributes to and interacts with cancer effects on the brain, and specifically, neuroinflammation [8]. This oversight is probably driven by the pressures of quick scientific turn-around, maintenance of the *status quo*, and budget constraints. As a consequence, it is currently unknown which, or the extent to which, inflammatory mediators may be amplified in tumor-bearing *aged* rodents. Thus, to address this oversight, the current study examined the neuroinflammatory phenotype in response to a mammary tumor in ovary-intact young adult mice and ovariectomized aged mice.

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2. Materials and methods

Animals. Young adult (3–4 month) and aged, ovariectomized (16–17 month) nulliparous female Balb/c mice (National Institute of Aging (NIA), Charles River, Wilmington, MA) were group housed (3–5/cage) and acclimated to the temperature-controlled (22 ± 1 °C) vivarium for 6 days under a 14:10 light:dark cycle (lights off at 15:00 h). Aged mice underwent ovariectomy to model post-menopausal circulating estrogen status. Rodent chow (Harlan 7912) and water were available *ad libitum* throughout the study and cotton nestlets and plastic huts were provided for nesting. All experiments were approved by the Ohio State University Institutional Animal Care and Use Committees and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [12]. All efforts were made to minimize animal suffering and to reduce the number of mice used. This project consisted of four treatment-balanced experimental replications. For a timeline of all experimental procedures, see Fig. 1.

Cells. The murine mammary non-metastatic 67NR cancer cell line, originating from a spontaneous mammary adenocarcinoma in a Balb/c mouse [13,14], was generously provided by Drs. Fred Miller and Lisa Polin at Karmanos Cancer Institute. Cells were grown in DMEM with 10% FBS, 2 mM L-glutamine, and 1 mM non-essential amino acids at 37 °C with 5% CO2 [15].

Experimental Procedures. Non-metastatic, syngeneic, orthotopic mammary tumors were induced in half of each age group. To control for the effects of surgery, tumor-free control mice underwent the same surgical protocol, but received an injection of phosphate-buffered saline (PBS) instead of cancer cells. Briefly, under anesthetization (isoflurane vapors), a 5-mm subcutaneous incision was made medial to the fourth nipple and 5×10^6 cells (in matrigel), or an equivalent volume of PBS for tumor-free controls, were injected into the associated mammary fat pad [15]. Incisions were closed with wound clips. Ear notches were placed for individual identification purposes. Body mass and tumor dimensions were measured twice/week. When tumors in the tumor-bearing mice were ~0.75 cm^2, mice were perfused with phosphate-buffered saline (PBS) to remove circulating leukocytes following deep CO_2 asphyxiation, and tissue (blood, brain, tumor, spleen) was collected. Immediately before perfusing, blood was collected via cardiac puncture through heparin-lined syringes for circulating cytokine quantification. Blood was stored on wet ice before being spun down at 2500 rpm for 20 min at 4 °C and plasma was stored at −80 °C. Hippocampal tissues were dissected and flash frozen in dry ice for later gene and protein expression quantification. Spleen and tumors were removed aseptically and weighed.

Immune PCR Gene Array. Total RNA was extracted from a random subset of the hippocampus (n = 6/group) using Qiagen RNeasy mini kits (CA, USA). RNA concentrations were measured and 260/280 ratios were determined to be 1.8–2.0 (NanoDrop, DE, USA). Five hundred ng of isolated RNA was synthesized to cDNA using the RT^2 First Strand Kit (SABiosciences/Qiagen, Frederick, MD, USA). Gene expression was measured using a real-time quantitative reverse transcription PCR array with primers designed to measure 84 mouse genes associated with mouse innate and adaptive immune response (SABiosciences/Qiagen; Cat. No. PAMM-052ZE-4) as previously described [16]. Gene expression was normalized using the geometric mean of housekeeping genes that did not vary among treatment groups, beta-2 microglobulin (*B2m*) and beta-glucuronidase (*Gusb*). Relative gene expression of individual samples was calculated by the comparative CT method ($2^{-\Delta\Delta CT}$). Specific pairwise comparisons of fold-regulation were analyzed using Qiagen's online free data analysis center, GeneGlobe, and heat maps were generated. Significant expression differences ($p < 0.05$) were organized into categories based on gene function and a weighted enrichment score was calculated (net sum of fold-changes for all genes in the category). Raw gene expression was also compared among all four treatment groups. Finally, a non-supervised hierarchical clustergram of the entire data set was generated (GeneGlobe) with dendograms indicating which treatment groups were most closely related based on gene expression profiles.

Plasma and Hippocampal Cytokine and Chemokine Concentrations. Circulating and hippocampal cytokines were measured from plasma or hippocampal homogenates using custom multiplex immunoassays (UPLEX, Meso Scale Discovery, Rockville, MD, USA) according to the manufacturer's instructions. To prepare the hippocampus for the assay, 0.2 mL of a sonication buffer containing 50 mM Tris base and a cocktail enzyme inhibitor (100 mM amino-n-caproic acid, 10 mM EDTA, 5 mM benzamidine HCl, and 0.2 mM phenylmethyl sulfonyl fluoride) was added to each sample. Each tissue was mechanically sonicated for ~20 s using an ultrasonic cell disrupter (Fisher Scientific, Pittsburgh, PA), centrifuged at 10,000×*g* at 4 °C for 10 min, and supernatants removed and stored at 4 °C until ELISA was performed within 1–2 d. Bradford protein assays were performed to determine total protein concentrations in each sample. Protein levels of IL-1β, IL-2, IL-6, IL-10, IFN-γ, TNF-α, CCL2, CCL3, CXCL1, CXCL2, and CXCL10 are presented as pg/100 μg of total hippocampal protein and pg/mL for plasma samples.

Statistical Analyses. Statistical analyses were conducted using StatView and Prism software. Two-way ANOVAs were used, with age and tumor treatment as independent variables. Where appropriate, Fisher's LSD post-hoc tests were conducted to reveal pairwise differences

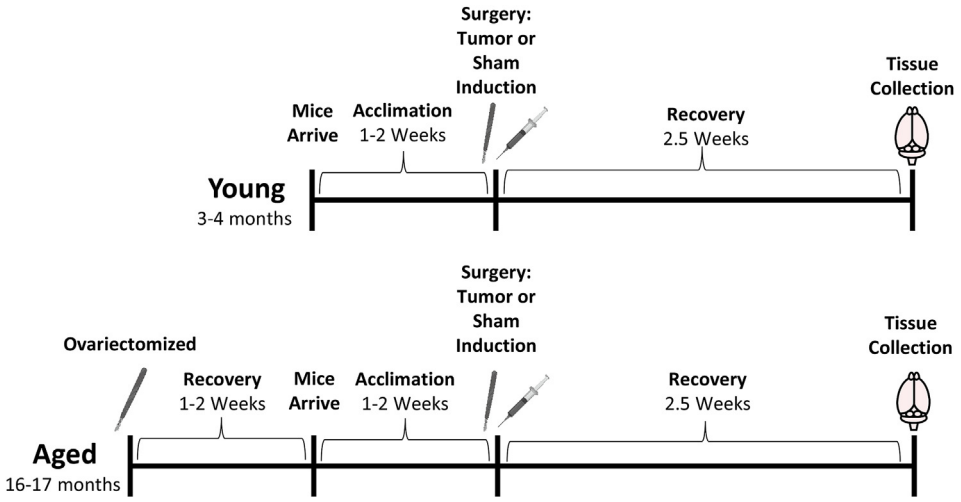

Fig. 1. Experimental timeline for each replicate of young and aged mice. Aged mice were ovariectomized prior to arrival. Tumor-bearing mice were surgically inoculated with mammary tumor cells while tumor-free control mice underwent a sham surgery with a PBS inoculation. Syringe and scalpel art created with BioRender.

between groups. In cases where only two groups were being compared, two-tailed t-tests were employed. For t-tests between young and aged tumor groups, tumor mass was used as a covariate to control for potential differences in tumor burden between young and aged mice, except for PCR array analyses for which only a subset of mice with equivalent tumor masses were used (young tumor: 1.4 ± 0.1 g; aged tumor: 1.4 ± 0.1 g). Statistical significance for all tests was set at $\alpha = 0.05$.

3. Results

Body, tumor, and spleen masses. As expected, aging (plus ovariectomy) increased body mass regardless of tumor treatment (Fig. 2A; $F_{1, 167} = 174.7$, $p \leq 0.0001$). In contrast, the presence of a tumor did not affect body mass ($p > 0.05$). Tumor growth was reduced in aged mice relative to young mice (Fig. 2B; $t_{88} = 3.606$, $p \leq 0.001$). Tumors increased both absolute spleen mass (data not shown) and spleen mass relative to body mass (relative spleen mass; $F_{1, 156} = 44.41$, $p \leq 0.0001$) in young, but not aged, mice (Fig. 2C; interaction $F_{1, 156} = 22.45$, $p \leq 0.0001$). Age increased relative spleen mass relative to respective tumor treatment-matched young controls, without a statistically significant overall main effect ($p > 0.05$).

Circulating cytokines and chemokines. Several patterns emerged in circulating inflammatory markers among groups. In support of our hypothesis, an interaction between aging and tumor treatment exacerbated the concentrations of circulating IL-2 in aged tumor-bearing mice (Fig. 3B; $F_{1, 37} = 3.943$, $p \leq 0.05$). Significant increases due to both aging and tumor treatments were likewise observed in circulating IL-10 (age $F_{1, 50} = 14.45$, $p \leq 0.0005$; tumor treatment $F_{1, 50} = 6.445$, $p \leq 0.05$) and TNF-α (age $F_{1, 50} = 14.63$, $p \leq 0.0005$; tumor treatment $F_{1, 50} = 17.96$, $p \leq 0.0001$); however, in both cases there were no significant interactions between age and tumor treatment (Fig. 3D, F; $p > 0.05$). Young mice drove the tumor-induced increase in IL-10 ($t_{24} = 4.535$, $p \leq 0.0005$), whereas the aged mice are primarily responsible for the tumor-induced increase in IL-2 ($t_{23} = 4.348$, $p \leq 0.0005$).

Alternatively, an interaction between aging and treatment significantly influenced concentrations of CCL2 (Fig. 3G; $F_{1, 35} = 6.425$, $p \leq 0.05$) such that tumors increased circulating concentrations in young mice, which were attenuated in aged mice even after controlling for age-differences in tumor mass (aged < young). Tumors increased IL-6 ($F_{1, 43} = 21.68$, $p \leq 0.0001$), CXCL1 ($F_{1, 49} = 41.17$, $p \leq 0.0001$), and CXCL10 ($F_{1, 35} = 12.49$, $p \leq 0.005$) with a tendency towards a similar attenuation in aged mice, though none of these reductions were statistically significant (Fig. 3C, I, K; $p > 0.05$).

Aging significantly elevated plasma concentrations of IFN-γ in both control and tumor-bearing mice ($F_{1, 49} = 13.53$, $p \leq 0.001$; Fig. 3E). Furthermore, aging increased MIP-1α protein concentrations ($F_{1, 34} = 9.545$, $p \leq 0.005$), which was primarily driven by the tumor-bearing groups. Among young mice only, the presence of a tumor decreased MIP-1α (Fig. 3H; $t_{16} = 4.424$, $p \leq 0.0005$). No changes in IL-1β and MIP-2 were observed among treatment groups (Fig. 3A, J; $p > 0.05$).

Brain immune qPCR array. In the hippocampus, gene expression of innate and adaptive immune factors were compared among treatment groups. Fold-changes in gene expression were calculated between relevant comparison groups (young tumor vs. young control, aged tumor vs. aged control, aged control vs. young control, old tumor vs. young tumor) and heat maps were generated (Fig. 4A–D). Significant expression differences ($p < 0.05$) were organized into categories based on gene function (Fig. 4A–D) and enrichment scores were generated based on these categories (Fig. 4A–D). Overall, the comparison with the greatest number of genes with significant fold-changes (and greatest absolute enrichment scores) was between hippocampi of aged and young tumor-bearing mice (Fig. 4D). For these comparisons, tumor mass was used as a covariate given that aged mice had smaller tumors than young mice. The next largest differences were observed in the comparison of aged hippocampi with and without tumors (Fig. 4B), then between young mice with and without tumors (Fig. 4A), and finally, very few fold-changes in immune gene expression were observed between aged and young control mice (Fig. 4C).

Of the five immune genes differentially expressed between hippocampi of young mice with and without tumors, all were increased with tumors (Fig. 4A). For example, mammary tumors increased both *Icam* (gene for leukocyte and endothelial cell glycoprotein that helps transmigration of leukocytes; $t_{10} = 2.7$, $p < 0.05$) and *Cd80* (cell membrane protein involved in T-cell activation and cytokine production; $t_{10} = 2.5$, $p < 0.05$) mRNA in the hippocampi of tumor-bearing young mice relative to young controls; these tumor effects were absent in aged mice (Fig. 4A; $p > 0.05$ in both cases).

Of the eight genes differentially expressed between hippocampi of aged mice with and without tumors, all but one (*Ccl12*) decreased with a tumor (Fig. 4B). For example, the chemokine, *Ccl12*, was significantly elevated by a mammary tumor in aged mice (not young mice; $t_8 = 2.4$, $p < 0.01$), whereas a transcription factor that binds to inflammatory gene promotors, *Stat4* ($t_9 = 2.4$, $p < 0.05$), and *Il-18* (cytokine in IL-1β family found in many brain cells; $t_{10} = 2.9$, $p < 0.05$) both decreased with a tumor in aged mice only (Fig. 4B).

Only one immune gene differed between aged and young tumor-free mice, with another gene that approached statistical significance; both encode enzymes and increased in aged hippocampi (Fig. 4C). Specifically, myeloperoxidase (*Mpo*), a neutrophil enzyme, tended to ($p = 0.06$) increase and caspase 1 (*Casp1;* enzyme that activates IL-1 and IL-18; $t_{10} = 2.9$, $p < 0.05$) increased with age in control mice (Fig. 4C); these age effects were absent among tumor-bearing mice.

All 21 genes differentially expressed between young and aged tumor-bearing mice decreased in aged tumor-bearing mice after controlling for tumor size (Fig. 4D). *Itgam* (marker of leukocytes; $t_{10} = 2.5$, $p < 0.05$), *Nfkb1* (part of the NF-κB transcription factor; $t_{10} = 4.6$, $p < 0.005$), *Tlr6* (toll-like receptor 6; $t_{10} = 2.9$, $p < 0.05$), and *Tyk2* (tyrosine kinase 2; $t_{10} = 3.3$, $p < 0.01$) are each examples of this pattern (Fig. 4D). In addition,

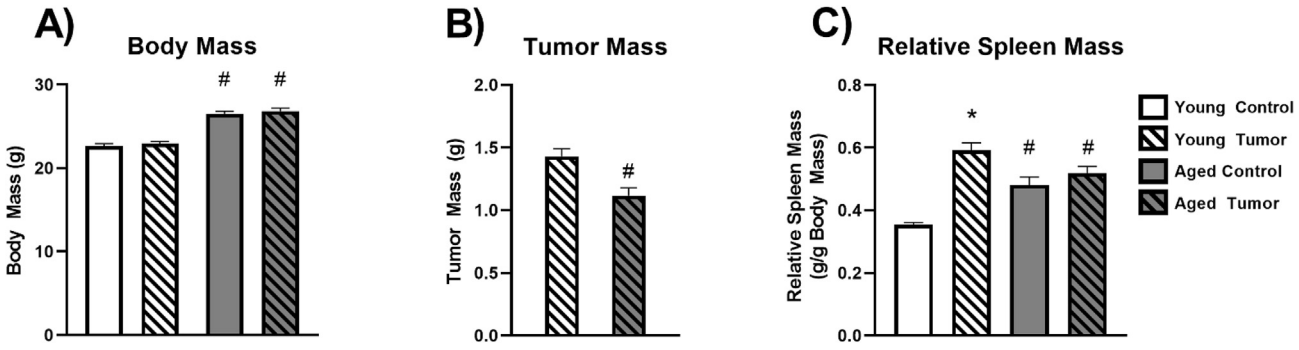

Fig. 2. Tissue mass comparisons among tumor and age conditions from all experimental replications. Mean ± SEM body mass (**A**), tumor mass (**B**), and spleen mass relative to body mass (**C**) at the time of tissue collection. $^{*}p \leq 0.05$ relative to age-matched tumor-free control mice; $^{\#}p \leq 0.05$ between age within tumor treatment group. n = 37–46 mice/group (all experiments combined).

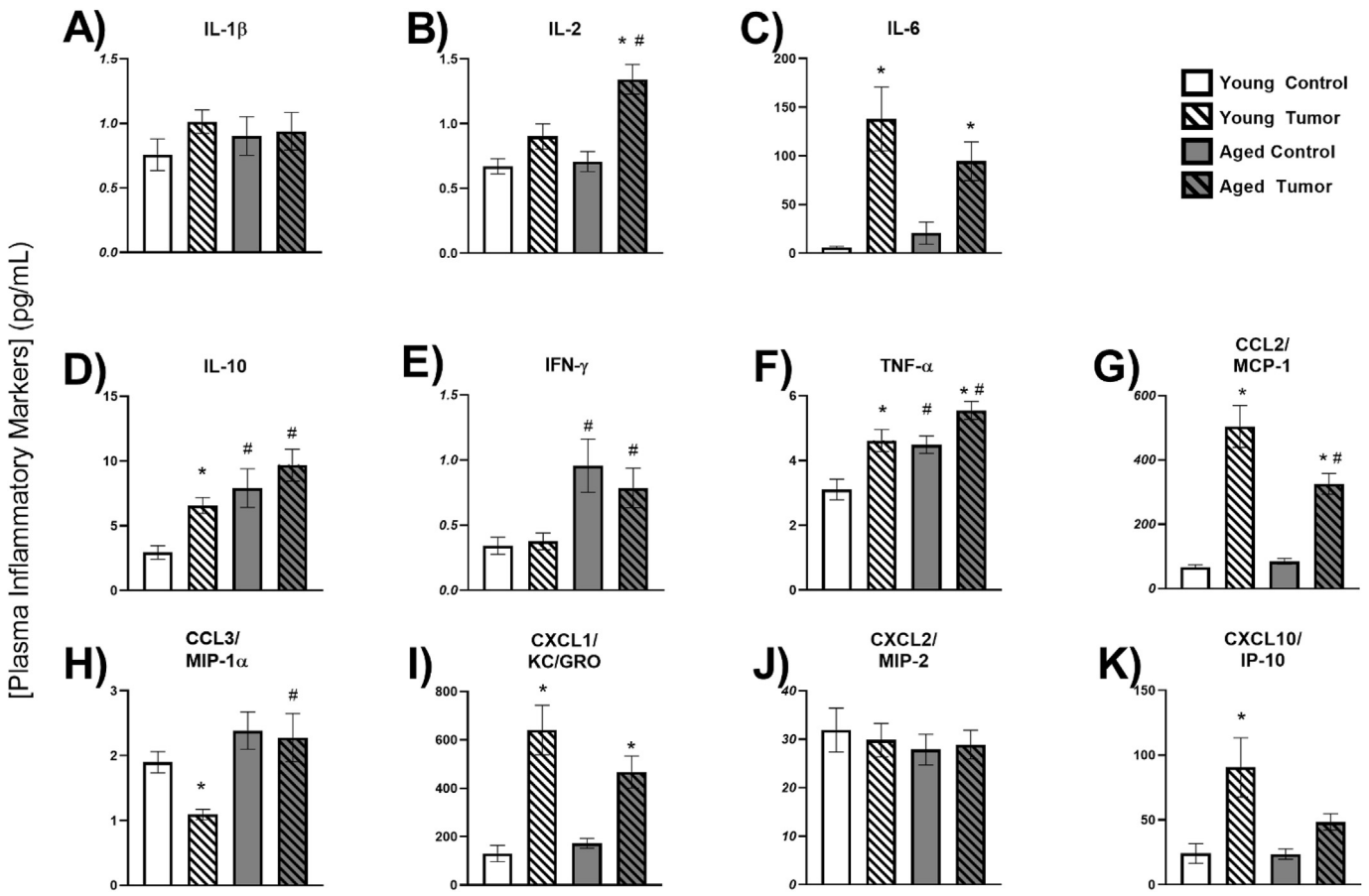

Fig. 3. Circulating plasma concentrations of IL-1β (**A**), IL-2 (**B**), IL-6 (**C**), IL-10 (**D**), IFN-γ (**E**), TNF-α (**F**), CCL2/MCP-1 (**G**), CCL3/MIP-1α (**H**), CXCL1/KC/GRO (**I**), CXCL2/MIP-2 (**J**), and CXCL10/IP-10 (**K**) in both tumor-free and tumor-bearing age groups. The data are presented as mean ± SEM. $^{*}p \leq 0.05$ relative to age-matched tumor-free control mice; $^{\#}p \leq 0.05$ between age within tumor treatment group. n = 9–16/group.

tumors decreased these four genes, and several others (*Irak1*, *Jak2*, *Il-18*, *Tlr6*, *Ly96*), relative to their age-matched tumor-free controls, indicating that not only did tumors decrease these immune genes in hippocampi of aged relative to young tumor-bearing mice, but also compared to hippocampi of aged healthy controls.

Hippocampal cytokine and chemokine protein. Protein of inflammatory markers were also quantified in the hippocampus and compared among treatment groups. Similar to that observed for hippocampal gene expression, significant interactions between age and tumor treatments resulted in tumor suppression of aging-induced elevations of inflammatory proteins. Aging-induced increases in IL-1β ($F_{1, 30} = 7.848$, $p \leq 0.01$), IL-2 ($F_{1, 31} = 6.189$, $p \leq 0.05$), IL-4 ($F_{1, 28} = 7.846$, $p \leq 0.01$), IL-6 ($F_{1, 30} = 7.401$, $p \leq 0.05$), IL-10 ($F_{1, 28} = 8.283$, $p \leq 0.01$), and CXCL10 ($F_{1, 32} = 4.614$, $p \leq 0.05$) were all significantly attenuated by mammary tumors (Fig. 5A–E, 5J). In a related pattern, hippocampal CXCL1 protein concentrations were elevated by tumors in young mice and aging attenuated this increase (Fig. 5H; $F_{1, 31} = 6.295$, $p \leq 0.05$). Aging, independent of tumor treatment, increased CCL3 (Fig. 5G; $F_{1, 34} = 18.35$, $p \leq 0.0005$) and CXCL2 (Fig. 5I; $F_{1, 33} = 21.14$, $p \leq 0.0001$) regardless of tumor treatment. Finally, CCL2 hippocampal protein was unaffected by age or treatment (Fig. 5F; $p < 0.05$).

4. Discussion

As expected and consistent with previous findings, aged female mice weighed more than younger adults [17] and mammary tumor growth did not affect body mass at these tumor sizes [15]. Increased body mass in the aged mice is likely due to increased adipose tissue [18]. Similar to previous studies [15,16], splenomegaly was observed in young, tumor-bearing mice in the present study and is likely driven by an accumulation of immune cells in the spleen [19]. Of note, this tumor-induced splenomegaly (absolute and relative to body mass) was masked in aged mice, in part, by an increase in spleen mass due to aging. Few studies have compared spleen mass between young and aged mice and existing results appear to be mixed ([20,21]; L. Pyter unpublished findings in other models). All mice were housed in the same room and received from the same supply center (NIA), thus it is unlikely that spleens in aged mice were enlarged due to ongoing infection. Finally, tumor mass was reduced in aged mice relative to young mice due to a slower rate of growth. This reduced tumor burden in aged mice is consistent with some mouse mammary tumor models [22,23], but contrary to other models [24–26]. Indeed, mounting evidence indicates that tumors elicit weaker immune responses in aged mice, which subsequently reduces growth and angiogenic factors used to support tumor growth, and thereby result in slower tumor growth [27]. In humans, while breast cancer incidence significantly increases with age, severity and recurrence decreases [28,29]. Indeed, reduced growth of breast cancers in aged women is associated with few tumor-infiltrating lymphocytes [30].

Previous studies indicate that both tumors (outside of the brain) and aging independently elevate circulating and brain markers of inflammation (reviewed in Refs. [6,31]). This neuroinflammation is associated with negative behavioral consequences, including impaired cognitive performance, fatigue, and affective-like behaviors. In addition, numerous experimental paradigms that integrate multiple inflammatory triggers, or "two-hits" (e.g., obesity plus bacterial challenge), report additive or synergistic effects of the triggers upon neuroinflammation [32–35]. Thus, we predicted that adding a peripheral tumor to an aged phenotype

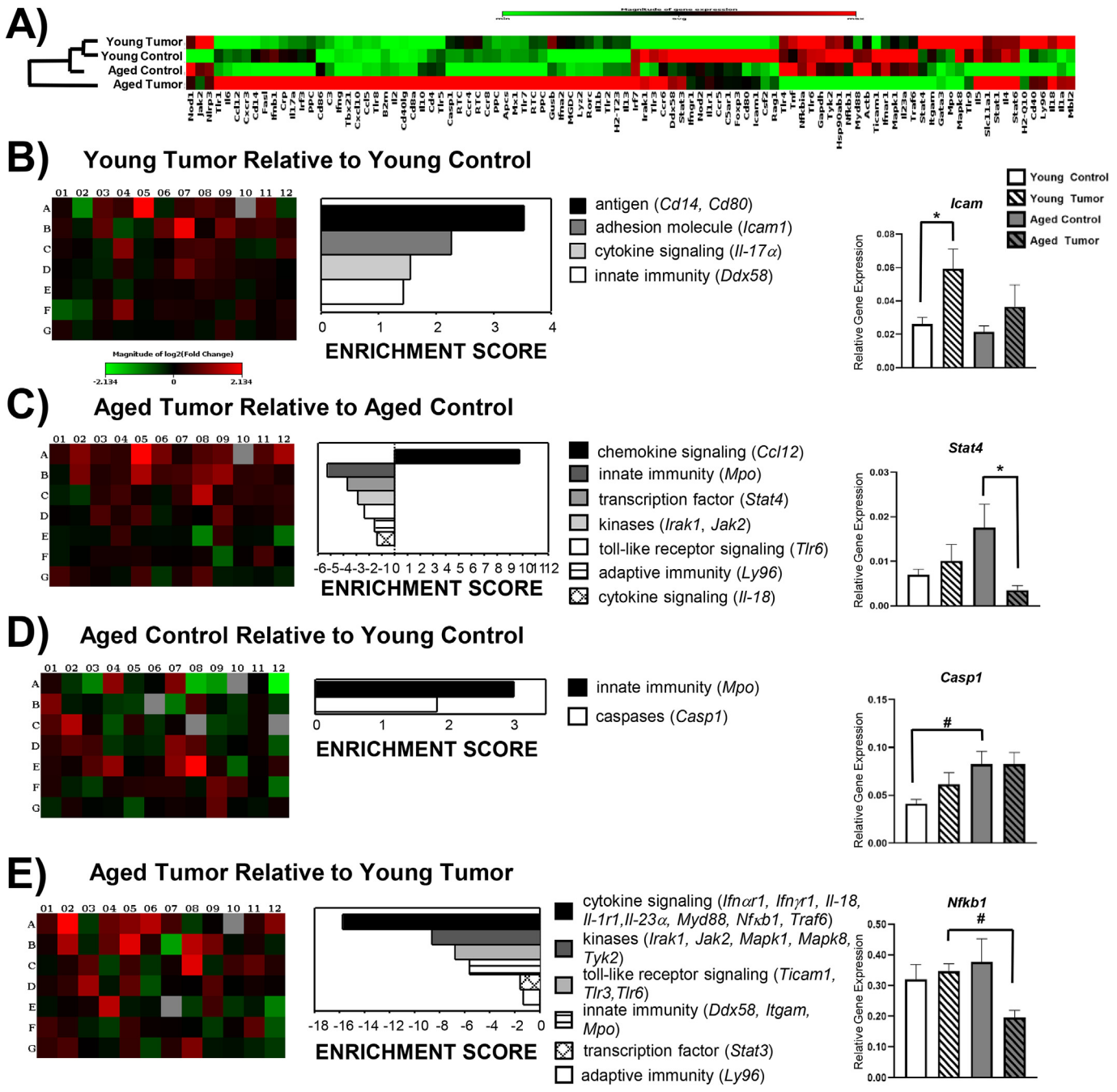

Fig. 4. Transcriptional profiling of hippocampus among tumor and age conditions. **(A)** Non-supervised hierarchical clustering of entire data set with associated heat map and dendograms indicating similar profiles among groups. The $\log_2$ transformed fold-change heat map for all genes quantified and associated enrichment scores (sum of fold-regulation for all significantly different genes [$p < 0.05$; listed]) based on their functional category comparing **(B)** young tumor-bearing mice relative to young controls, **(C)** aged tumor-bearing mice relative to aged controls, **(D)** aged controls relative to young controls, and **(E)** aged tumor-bearing mice relative to young tumor-bearing mice. Representative bar graphs of these gene expression (relative to geometric mean of *B2m* and *Gusb* endogenous control genes) patterns are shown including all four treatment groups. The data are presented as mean ± SEM. $^{*}p \leq 0.05$ relative to age-matched tumor-free control mice; $^{\#}p \leq 0.05$ between age within tumor treatment group. n = 6/group.

would exacerbate neuroinflammation. In contrast, tumors consistently suppressed aging-induced inflammation.

Protein levels of circulating inflammatory mediators revealed a couple of interesting patterns. First, consistent with our hypothesis, IL-2, IL-10, IFN-γ, TNFα, and CCL3 levels were exacerbated in tumor-bearing aged mice compared to tumor-bearing young adult mice. These findings are consistent with what others have reported in aged mice following a peripheral immune challenge [36,37]. Second, and contrary to our hypothesis, IL-6, CCL2, CXCL1, and CXCL10 expression levels were elevated with a tumor in young adult mice compared to tumor-free young mice, but this elevation tended to be blunted in the tumor-bearing aged mice. While unexpected, these findings are not surprising given the fact that various inflammatory mediators are known to have different kinetics as a function of age [38]. Similarly, age-associated alterations in macrophage signaling are pathway-specific, with advanced age impairing TLR-mediated pathways, specifically [39]. These findings indicate that mammary tumors do not evoke a uniform inflammatory response in the circulation of young and aged mice.

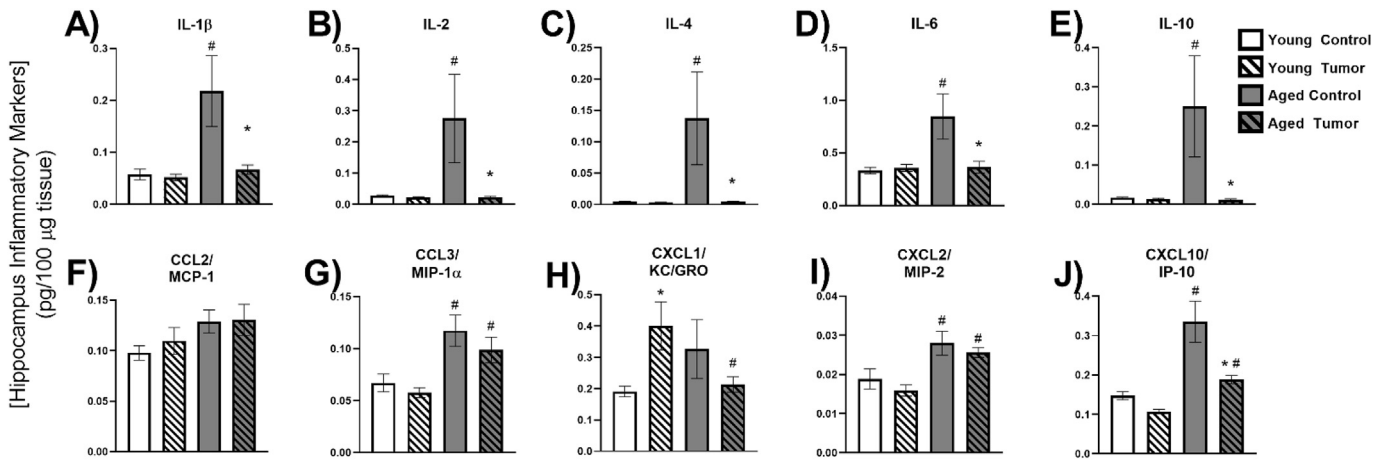

Fig. 5. Mean ± SEM protein expression of inflammatory markers within the hippocampus of both age groups of tumor-free and tumor-bearing mice: IL-1β (**A**), IL-2 (**B**), IL-4 (**C**), IL-6 (**D**), IL-10 (**E**), CCL2/MCP-1 (**F**), CCL3/MIP-1α (**G**), CXCL1/KC/GRO (**H**), CXCL2/MIP-2 (**I**), and CXCL10/IP-10 (**J**). $^{*}p \leq 0.05$ relative to age-matched tumor-free control mice; $^{\#}p \leq 0.05$ between age within tumor treatment group. n = 6–12/group.

Differences in tumor mass between young and aged tumor-bearing mice did not drive any hippocampal transcriptional differences, as tumor mass was held constant between groups when selecting brain tissue samples for the PCR array. Consistent with previous studies of this peripheral tumor model [15,40] and other cancer models [9,10,41,42], tumors increased immune gene expression related to both antigen presentation and inflammation in the hippocampi of young rodents. Except for *Icam* [40], all five other genes were uniquely identified in the present study using the PCR array approach (*Cd14, Cd80, IL-17a, Ddx58*). Some previously identified genes that were elevated using this tumor model were absent here, possibly due to the gene array chemistry (SYBR green), their absence on the array, and/or the reporting criteria requirement of fold-changes instead of more subtle differences identified with absolute gene expression. Similarly, aging increased previously reported *Casp-1* [43] and *Mpo* [44] gene expression in the hippocampus. Of note, aging did not confer abundant hippocampal transcriptional changes using this "innate and adaptive immune response" targeted array, compared with full microarray analyses of the whole brain [34].

Most relevant to our overall objective, however, were the changes in immune transcription in the hippocampus when a tumor was added to an aged background in relation to either aged controls or young tumor-bearing mice. Indeed, the overwhelming pattern was clearly summarized by the hierarchical clustering analysis, in which aged mice with tumors were least related to all three other treatment groups. In contrast to our prediction, tumors in aged mice overwhelmingly *decreased* immune gene expression relative to both aged controls and young tumor-bearing mice (except for *Ccl12*), from genes involved in cytokine and toll-like receptor signaling, to immune-related kinases and transcription factors. A robust suppression in the neuroinflammatory response following two "hits", however, is not entirely unprecedented. Likening aging plus tumors to a "two-hit" model of inflammation, decreased immune transcription was also recently observed in another "two-hit" paradigm using this model: tumors plus peripheral immune stimulation (i.p. injection of lipopolysaccharide [LPS]; [45]). In this paradigm, tumors similarly reduced neuroinflammatory transcription to a peripheral LPS injection. These tumor-induced reductions in inflammatory brain transcription may reflect previously reported insensitivity of brain astrocytes to IL-4 or IL-10 stimulation [46,47] and the downregulation of CX3CR1 on microglia after LPS [48,49] in aged Balb/c mice. Indeed, a peripheral tumor, which produces inflammatory signals, may be considered a chronic, progressing peripheral inflammatory "challenge." Similarly, sensitization with methamphetamine administration followed by an LPS challenge results in a robustly suppressed transcriptional neuroinflammatory response in the hippocampus of young adult mice [50]. Furthermore, methamphetamine withdrawal combined with an effortful behavioral task suppresses neuroinflammation in the fronto-cortical region of the brain of young adult rats [51].

Hippocampal protein expression of nearly all inflammatory markers examined (IL-1, IL-2, IL-4, IL-6, IL-10, CCL3, CXCL2, CXCL10) was robustly increased in tumor-free aged mice compared to young tumor-free control mice, consistent with what others have reported in Balb/c mice [52,53]. Expression of these inflammatory mediators in tumor-bearing aged mice however, was profoundly suppressed compared to age-matched control mice, similar to the observed patterns of gene expression. The protein data confirm that these results run counter to our hypothesis and the majority of the "two-hit" literature demonstrating that the interaction of two inflammatory insults *exacerbates* the neuroinflammatory response [33,34,54,55], which in turn affects synaptic plasticity and various behavioral impairments [1]. The reduced estrogens in the ovariectomized, aged mice may account for some neuroinflammatory increases in aged versus young adult mice, as estrogens are generally anti-inflammatory [56,57]. However, this logic would not directly account for the remarkable differences between aged tumor-bearing and tumor-free mice. Taken together with the transcriptional data, whether an exacerbated or suppressed neuroinflammatory response is evoked may depend on the nature of the two insults/challenges, and the resulting activated signaling pathways. In the case of a tumor, already strained energetic resources in an aged model (due to immunosenescence) may be shunted towards energetically-expensive tumor growth, resulting in a deficiency of resources to mount a proper neuroinflammatory response. Importantly, it should be noted that reduced constitutive brain cytokines are just as deleterious for synaptic plasticity and neural functions as elevated responses [35,51,58,59]. In summary, these findings suggest that peripheral tumors, in the context of advanced age, interfere with the normal central immunity. Future studies will directly examine whether this response interferes with appropriate responding to danger signals from the peripheral immune system.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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