Published in final edited form as:

Glia. 2015 May ; 63(5): 754–767. doi:10.1002/glia.22782.

Aging-like Changes in the Transcriptome of Irradiated Microglia

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Abstract

Whole brain irradiation remains important in the management of brain tumors. Although necessary for improving survival outcomes, cranial irradiation also results in cognitive decline in long-term survivors. A chronic inflammatory state characterized by microglial activation has been implicated in radiation-induced brain injury. We here provide the first comprehensive transcriptional profile of irradiated microglia. Fluorescence-activated cell sorting (FACS) was used to isolate CD11b⁺ microglia from the hippocampi of C57BL/6 and Balb/c mice 1 month after 10Gy cranial irradiation. Affymetrix gene expression profiles were evaluated using linear modeling, rank product analyses. One month after irradiation, a conserved irradiation signature across strains was identified, comprising 448 and 85 differentially up- and down-regulated genes, respectively. Gene set enrichment analysis (GSEA) demonstrated enrichment for inflammation, including M1 macrophage-associated genes, but also an unexpected enrichment for extracellular matrix and blood coagulation-related gene sets, in contrast previously described microglial states. Weighted gene co-expression network analysis (WGCNA) confirmed these findings and further revealed alterations in mitochondrial function. The RNA-seq transcriptome of microglia 24h post-radiation proved similar to the 1-month transcriptome, but additionally featured alterations in apoptotic and lysosomal gene expression. Re-analysis of published aging mouse microglia transcriptome data demonstrated striking similarity to the 1 month irradiated microglia transcriptome, suggesting that shared mechanisms may underlie aging and chronic irradiation-induced cognitive decline.

Keywords

brain radiation; inflammation; hippocampus; extracellular matrix; aging

INTRODUCTION

Radiation therapy is essential in the management of primary and metastatic brain tumors. Although it improves survival, radiation causes progressive deficits in memory, attention, and information processing that are most severe in pediatric patients, but also decrease quality of life in adult long-term survivors (Gibson and Monje 2012). Current evidence

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points to dysfunctional endogenous neurogenesis, altered neuronal plasticity, and progressive diffuse white matter degeneration as major contributors to cognitive impairment after irradiation (Padovani et al. 2012). These defects may result in part from chronic activation of microglia, the resident macrophages of the central nervous system (CNS) (Monje et al. 2002; Monje et al. 2003).

The effects of microglial activation are manifest in CNS trauma, stroke, neurodegenerative disease and psychiatric illness, and contribute to neurocognitive aging (Blank and Prinz 2013; Cunningham 2013; Saijo and Glass 2011; Zhang et al. 2013b). Although activated microglia can generate neurotoxic cytokines, they may also serve neuroprotective functions (Hanisch and Kettenmann 2007; Mosser and Edwards 2008). As such, a deeper understanding of microglial behavior is critical to the development of therapies that combat the late effects of cancer therapy. Radiation leads to the chronic activation of microglia, with persistent activation associated with progressive cognitive decline and ablation of neurogenesis even decades post-irradiation in humans (Monje et al. 2007). Despite the central role of microglia to neurological diseases, very few studies have evaluated the transcriptome of microglia from diseased brains, and no transcriptome data are available for microglia isolated following brain irradiation.

As such, we performed comprehensive transcriptome analysis of acutely isolated hippocampal microglia, one month after 10Gy or sham cranial irradiation. The hippocampus was selected given its critical role in learning and memory and the presence of hippocampal neurogenesis, all of which are permanently impaired by brain irradiation (Monje et al. 2002; Monje et al. 2003). We evaluated C57BL/6 and Balb/c mice, which have contrasting pro-inflammatory (Th1-biased) and anti-inflammatory (Th2-biased) phenotypes, respectively (Guyot et al. 2013), but are both well documented to exhibit impaired neurogenesis after cranial irradiation (Lee et al. 2013; Mizumatsu et al. 2003). We hypothesized that gene expression patterns may differ between these two strains but that a common irradiation-induced signature could be identified. We here demonstrate that the 1-month irradiated microglia transcriptome is characterized by changes in inflammation, extracellular matrix and mitochondrial gene expression. Moreover, these changes, which we find to be already present within 24h of irradiation, become strongly enriched in microglia during the aging process.

MATERIALS AND METHODS

6.5 week-old female C57Bl/6 and Balb/C mice were anesthetized and irradiated with 0 or 10Gy cranial irradiation, shielding the eyes, ears and nose. After 24h or 1 month, hippocampi of anesthetized, PBS-perfused mice were removed and dissociated with Papain and DNase. Viable (PI-) microglia (CD11b+) were sorted by fluorescence-assisted cell sorting (FACS) into Trizol. Amplified cDNA or cDNA libraries were then generated and used for comprehensive transcriptome evaluation of 24h or 1 month samples using Affymetrix 43 20.0 microarrays or RNA seq, respectively. Bioinformatics analysis was performed using R/Bioconductor, gene expression commons (GEXC), gene set enrichment analysis (GSEA), ingenuity pathway analysis (IPA,) and weighted gene co-expression network analysis (WGCNA) as appropriate. Differential expression of a subset of genes was

confirmed via quantitative PCR using Taqman Primers. RNAseq data from 100bp paired-end reads were mapped using Tophat2 with Bowtie2 and assembled using Cufflinks. Differential expression analysis was performed using Cuffdiff. Microarray data reported here are MIAME compliant and deposited in the Gene Expression Omnibus (GEO) public repository (GSE55968). The raw RNA-seq data have been deposited in the sequence read archive (SRA; PRJNA# 268981). Full details of materials and methods employed are available as online supplemental methods.

RESULTS

To evaluate the transcriptome of microglia 1 month after irradiation, FACS sorted viable CD11b+ microglia were collected from freshly dissociated hippocampi of adult C57BL/6 and Balb/c mice 1 month after 10 Gy or sham cranial irradiation. RNA from 3 animals in each group was used for microarray analysis to obtain comprehensive transcriptome data for Balb/c and C57BL/6 irradiated and unirradiated microglia (total of 12 arrays) (Fig. 1).

Strain differences in baseline microglial gene expression

Baseline (sham irradiated) microglia gene expression between C57BL/6 and Balb/c mice was evaluated using a significance threshold of corrected $P < 0.05$ and >2 -fold change. 694 genes were significantly differentially expressed between strains (Sup. Table 2). As expected, the baseline expression of many inflammation-related genes differed between the two strains, including immune-related receptors (e.g. Ccr6, Tlr4, Il7r, P2rx7), inflammatory cytokines (e.g. Il6, Il15), and major histocompatibility complex genes (H2-Aa, H2-Dma, H2-D1, H2-K1, H60a). To probe the functional differences in gene expression between the two mouse strains, we used the popular bioinformatics tool GSEA. GSEA identifies the enrichment or depletion of predetermined gene sets on the basis of differences in gene expression between two experimental conditions (Subramanian et al. 2005). From the Broad Institute's Molecular Signatures Database (MSigDB), we used annotated gene sets from the Gene Ontology (GO) project (which groups genes by molecular function, cellular component, or biological process) and from curated pathway databases including Biocarta, Reactome, and the Kyoto Encyclopedia of Genes and Genomes (KEGG). Using this analysis, the non-irradiated C57BL/6 and Balb/c microglia showed no significant enrichment or depletion for any gene sets. Thus, although gene expression differences between Balb/c and C57BL/6 microglia exist at baseline, these differences did not appear to be coordinated in established biological processes or pathways.

Irradiated microglia transcriptome

Gene expression of irradiated microglia was compared between C57BL/6 and Balb/c mice. 639 genes were significantly differentially expressed between strains (Sup. Table 3). As seen for baseline expression patterns, there was no significant enrichment or depletion for any gene sets from the GO project or pathway databases. Of these 639 genes, 417 were in common with the 694 differentially expressed genes between strains in the sham-irradiated microglia, suggesting that the majority of strain-dependent gene expression differences are unaffected by irradiation.

We next compared the transcriptional changes in microglia between 10 Gy irradiated and sham irradiated mice at 1 month. In C57BL/6 mice 233 genes were significantly differentially expressed after irradiation, and in Balb/c mice 305 genes were differentially expressed (Sup. Tables 4, 5); 76 genes were significantly differentially expressed in both Balb/c and C57BL/6 mice, suggesting a significantly overlapping response (P -value $< 2.2 \times 10^{-16}$, Fisher's exact test) (Fig. 2a). Of genes that were significantly differentially expressed in only 1 strain, the direction of differential expression was preserved between strains for most genes (data not shown).

To better evaluate the interaction between strain and irradiation response, we generated a linear model accounting for both mouse strain and radiation treatment and found only 8 genes that responded to radiation significantly differently between strains (Fig. 2b, Sup. Table 6). Of these 8 genes, 4 (Il6, Ifi44l, H60a, and Oasl2) are included in GO annotations for immune response, suggesting subtle differences in microglial immune signaling in response to irradiation between strains. Remarkably, Balb/c microglia had an over 7-fold higher induction of pro-inflammatory interleukin-6 expression than C57BL/6, though the gene was upregulated in both strains in response to irradiation. Overall, the very small number of genes responding differently to irradiation between strains suggested a conserved microglial response to irradiation. To further evaluate this, we performed principal component analysis of the expression data and found the first principal component divided the 12 mice by strain, while the second principal component divided mice into irradiated and sham groups (Fig. 2c). Moreover, the same groups of genes are differentially expressed in both strains (Fig. 2d). Taken together, these analyses suggest that C57BL/6 and Balb/c microglia share a highly conserved response to irradiation.

Given this conserved response, data were pooled for both strains of mice to identify a conserved signature of irradiation. For this analysis, we used the Rank Product method, which uses ranks of fold changes in each strain to identify genes with conserved differential expression in both strains, thereby increasing the power of identification. Rank Product analysis detected 448 significantly upregulated (Sup. Table 7) and 85 significantly down-regulated genes (Sup. Table 8) for a total of 533 differentially expressed genes (fold change ≥ 2 and false discovery rate ≤ 0.05). Henceforth, this set of genes will be referred to as the 1-month irradiated microglia transcriptome. To test the generalizability of the transcriptome, which was obtained using 3 mice from each radiation treatment group of each strain, we performed qPCR for a selection of differentially expressed genes, as well as others not differentially expressed, but implicated in inflammatory signaling and microglial identity or polarity using all mice in each group (Sup. Fig. 4). Of 17 tested genes from the irradiated microglia signature, 15 were significantly differentially expressed by qPCR in at least 1 strain with 11 of these being differentially expressed in both strains. The average direction of differential expression by PCR agreed with that of the microarray in all 17 cases. Ccl12 was significantly upregulated by PCR but was absent in the microglial transcriptome only because its corresponding probe set was not annotated in the array annotation package used in R. IL1b missed significance for inclusion in the irradiated microglia transcriptome, but proved significant when more animals were evaluated by qPCR. These findings suggested

that the number of animals used for our array analysis yielded a conservative rather than an exhaustive irradiated microglia transcriptome.

To interpret the functional significance of the 1-month irradiated microglia transcriptome, we used the GSEA approach to look for coordinated patterns of differentially expressed genes. Because of the substantial overlap between many gene sets in the GO project or pathway databases, to aid in interpretation of these results, we constructed networks to group together overlapping gene sets. Carrying out GSEA using GO terms, we found enrichment for inflammation-related signaling, including various chemokine and cytokine gene sets as expected; however, the most significantly enriched gene sets were related to extracellular matrix (ECM). There was also enrichment for gene sets relating to cation homeostasis, caspase activity, protease inhibitor activity, skeletal development, cell structural components, and cell junctions (Fig. 3a). GSEA using pathway database gene sets, again revealed enrichment for cytokine and chemokine signaling, as well as for coagulation cascade-related gene sets among various metabolic pathways (Fig. 3b). Full listings of gene ontology and pathway gene sets significantly enriched in the 1-month irradiated microglia transcriptome are provided as Supplementary Tables 9 and 10 respectively. No GO project or pathway database gene sets were significantly enriched in the sham-irradiated microglia relative to the irradiated microglia. Repeating GSEA with the individual mouse strains revealed highly similar results (data not shown).

Microglial phenotype after irradiation

To better understand the chronic microglial activation state, we compared the 1-month irradiated microglia transcriptome to two macrophage states: M1 or classical polarization (pro-inflammatory) and M2 or alternative polarization (immunomodulatory). We used GSEA to determine the enrichment for the M1- and M2-associated genes using custom gene sets adapted from human macrophage gene modules identified by Xue et al. (2014) (Sup. Table 11). Using GSEA, we found that M1-associated genes are significantly enriched in irradiated microglia ($P < 0.001$ Fig. 4a). Contrastingly, the M2-associated genes were not significantly enriched ($P = 0.291$). This finding was consistent when the mouse strains were analyzed separately. The M1-like microglia phenotype is further supported by the finding that the top four predicted upstream regulators identified by Ingenuity Pathway Analysis based on the irradiated microglia transcriptome were lipopolysaccharide, IL6, TNF, and IL1B, which all promote pro-inflammatory M1 polarization of macrophages (Murray and Wynn 2011). Interestingly, examination of the subset of M1 and M2 genes most commonly used to phenotype activated macrophages in literature did not suggest either polarization state was established following irradiation (Fig. 4b).

We expected that the irradiated microglia transcriptome would be more M1-like, as we had observed enrichment for many inflammation-related gene sets. However, enrichment for ECM and coagulation gene sets was unanticipated. To determine if these gene sets may be directly implicated with inflammatory processes, we asked whether M1 or M2 macrophages show enrichment for these other gene sets. GSEA of publicly available gene expression data for mouse M1 microglia and M2 macrophages failed to identify enrichment for any of the ECM or coagulation gene sets that were most highly enriched in irradiated microglia (data

not shown) (Dirscherl et al. 2010; Mullican et al. 2011). Therefore, despite evidence of some functional similarity to the M1 polarization state, irradiated microglia are defined by a unique transcriptional state that is distinct from previously established classical microglial polarization states.

Pathway and network analysis of irradiated microglia

Given that we found that 1-month irradiated microglia have an M1-like, yet distinct, activation profile, we explored the pathways altered in irradiated microglia using Ingenuity Pathway Analysis (IPA). We input into IPA all significantly differentially expressed genes between irradiated and sham-irradiated mice (adjusted p -value ≤ 0.05). We first used IPA to identify the enrichment of these genes for canonical pathways in the Ingenuity Knowledge Base (Fig. 5a). The most significantly enriched pathway was the hepatic fibrosis / hepatic stellate cell activation pathway, which is consistent with the increased ECM component of the irradiated microglia transcriptome. Other top canonical pathways, including granulocyte adhesion, atherosclerosis signaling, and pathogenesis of multiple sclerosis, are similarly related to inflammation.

We then used IPA to construct functional networks from our differentially regulated genes based on established direct and indirect gene interactions. The top network for the irradiated microglia transcriptome was composed of genes associated with DNA replication, recombination, and repair, cardiovascular disease and congenital heart anomalies (Fig. 5b). This network was centered on CEBPA, a significantly downregulated (2.4-fold decrease) DNA binding protein in the irradiated microglia transcriptome. The second and third networks, centered around the aryl hydrocarbon receptor and the cell cycle regulatory gene *Cdkn1a*, respectively, are provided in Supplementary Fig. 6.

Weighted gene co-expression correlation network analysis (WGCNA)

To further characterize the 1-month irradiated microglia transcriptome, we used the WGCNA method to identify modules of highly correlated genes in our gene expression data without *a priori* structural or functional knowledge (Langfelder and Horvath 2008). Analyzing our preprocessed and filtered microarray data from 12 mouse microglia samples, we identified 27 non-overlapping gene modules (arbitrarily labeled with color names), ranging from 36 to 1463 genes in size (Fig. 6a, Sup. Tables 12, 13). Three modules (green, pink, and red) were significantly correlated with radiation treatment ($p < 0.05$), while six modules (brown, yellow, black, dark turquoise, tan, and darkgreen) are significantly inversely correlated with radiation treatment ($p < 0.05$). The genes included in the 3 significantly associated modules accounted for 95% (426 / 448) of the upregulated genes in the irradiated microglia transcriptome (Fig. 6b, 7), though fewer than half of the genes in each module had been captured within the list of significantly upregulated genes with irradiation. Importantly, none of these three modules are correlated with mouse strain differences, further supporting that there is a conserved response to radiation in microglia between mouse backgrounds (Fig. 6a). Six modules (turquoise, blue, purple, darkorange, lightcyan, grey60, and black) were significantly correlated with strain differences (Fig. 6a).

We functionally analyzed the three modules highly correlated with radiation using DAVID to assess for enrichment of GO terms. From this analysis, we found that the green and red modules capture the ECM component of the irradiated microglia transcriptome, while the pink module captures the inflammation-related component (Fig. 6c, 6d, 6e). The green module also featured enrichment for gene sets related to contractile fibers, cell adhesion, neural development, and calcium ion binding. The pink module featured enrichment for plasma membrane, peptidase inhibitor, and calcium ion binding-related gene sets. Interestingly, the red module also featured enrichment for mitochondria-related gene sets, suggesting a possible change in microglial energy states—a finding only appreciated when the entire module was analyzed without filtering for significantly differentially expressed genes. Full listings of GO terms enriched in the green, pink and red modules, as determined by DAVID, are provided in Supplementary Tables 14, 15, and 16, respectively.

Since the 1-month irradiated microglia transcriptome is M1-like, we then asked if these three radiation-associated gene modules are enriched in M1-polarized microglia. Using GSEA, we assessed the enrichment for gene sets created from the three modules in a public M1 data set (Dirscherl et al. 2010). The pink module was significantly enriched in M1 ($p < 0.05$), while the green and the red modules were not. Thus, two of the three novel gene modules appear unique to radiation.

Transcriptome of microglia isolated 24 hours following cranial irradiation

Having characterized the persistent alterations in microglia activation 1 month following radiation, we then sought to understand the acute signaling events following cranial irradiation that precede this chronic microglial state. We repeated our isolation of C57BL/6 and Balb/c mice hippocampal microglia, except using mice 24 hours following 10 Gy or sham cranial irradiation (2 animals per group, 8 total). RNA-seq was used to obtain comprehensive transcriptome data. When comparing irradiated with sham-irradiated mice while pooling the strains, we found 436 significantly differentially expressed genes ($FDR < 0.05$, 246 upregulated and 190 downregulated) (Sup. Table 17). Seventy-nine of these genes overlap with the 1 month irradiated microglia transcriptome.

We used GSEA to assess the functional enrichment for GO terms and pathway database gene sets (Biocarta, Reactome, and KEGG) in the 24 hour irradiated microglia transcriptome. We found no significantly enriched GO terms; however, using pathway database gene sets, we found significant enrichment for gene sets relating to p53-family genes (p53, p63, and p73) and lysosomes ($FDR < 0.05$) (Sup. Table 18), consistent with known mechanisms of radiation-induced cell death (Persson et al. 2005). In addition, there was significant down-regulation of gene sets involved in chondroitin sulfate biosynthesis, the neuronal system, basal cell carcinoma, and glycosaminoglycan biosynthesis (Sup. Table 18).

To evaluate the similarity of the 24 hours irradiated microglia transcriptome with the 1-month transcriptome, we used GSEA to assess the enrichment for differentially-expressed genes in the green, red and pink modules in 1-month irradiated microglia as well as for human M1 and M2-polarized macrophage gene sets (Xue et al. 2014). Using GSEA, we found that the 24-hour transcriptome is significantly enriched for gene sets comprising the

green, pink and red modules of the 1-month transcriptome, as well as M1-polarized macrophage gene set, but not M2-polarized macrophages, in similar manner to the 1-month transcriptome. Overall, enrichment was strongest for the radiation-associated red module. (Fig. 8a and 8b).

Comparison with aging microglia gene expression

Normal aging is associated with decreased neurogenesis, gradual loss of cerebral volume and cognitive decline, all of which are observed following cranial radiation therapy. Moreover, changes in microglial phenotype have been associated with aging and may contribute to these aging-related changes in brain structure and function (Rozovsky et al. 1998; Sierra et al. 2007; Streit et al. 2004). We therefore asked whether or not the microglial features present 1 month after irradiation may be associated with normal aging. We re-analyzed a published RNA-seq dataset containing microglia isolated from 5-month and 24-month old C57BL/6 mice (Hickman et al. 2013). After generating a ranked list of differentially expressed genes between the two age groups (Sup. Table 19), we used GSEA to assess the enrichment for the gene sets related to the irradiated microglia transcriptome and human M1 and M2-polarized macrophage gene sets (Xue et al. 2014). We found significant enrichment ($FDR < 0.001$) for all 3 modules of the radiation response, as well as for M1 (Fig. 8c). These results suggest that the microglia transcriptome is similarly impacted by both normal aging and brain irradiation.

To assess the specificity of this observed similarity between aged and irradiated microglia, we compared the degree of enrichment for the radiation response in the aged microglia data to other gene sets from throughout the literature, as archived in three databases. The GeneSigDB (release 4), MSigDB (mouse), and MSigDB (human) collections contain 3515, 2101, and 8377 curated gene signatures respectively (Culhane et al. 2012; Liberzon et al. 2011). When all gene sets from these collections were included in a parallel analysis using GSEA, the aging microglia transcriptome was more highly enriched for the upregulated portion of the 1-month irradiated microglia signature than any other curated gene set. Consistent with this, analysis of the ranked gene list for aged microglia via GSEA for GO terms revealed significant enrichment for both both ECM and inflammation-related gene sets (Sup. Table 20).

DISCUSSION

Irradiated Microglia Transcriptome

This study provides the first genome-wide expression profile of irradiated microglia. Importantly, this transcriptome represents the state of microglia acutely isolated from the irradiated brain, not the transcriptome of in vitro cultured microglia. Transcriptional analysis from both C57BL/6 and Balb/c mice revealed a similarly persistent pro-inflammatory profile of microglia for at least 1 month following irradiation, with altered expression of numerous ECM-related and coagulation-related genes. This characteristic pattern of “chronic” changes was already detectable within 24 hours following cranial irradiation. Prior studies have evaluated expression changes for select genes after irradiation in dissected or whole brain samples, revealing a wave of differentially expressed genes modulating inflammation, signal

transduction and metabolism that largely return to normal within hours to days within bulk tissue populations (Baker and Krochak 1989; Hellstrom et al. 2011; Kalm et al. 2009; Lee et al. 2013; Zhao et al. 2006). Nevertheless, long-term activation of microglia following radiation has been consistently observed by immunohistochemistry (Conner et al. 2011; Monje et al. 2002). We here elucidate the signature of transcriptional changes that persist in chronically irradiated microglia, and find them to be similar to changes occurring in microglia during aging. As such, our findings may offer new insights into the pathophysiology of long-term cognitive decline following brain irradiation and aging.

The irradiated microglial transcriptome is pro-inflammatory with a significant M1-like component and robust expression of IL6 and TNF. In contrast to prior literature suggesting that M1-like changes resolve over time following injury, giving way to reparative M2-type processes, we found significant enrichment for M1 but not M2 genes at both 1 day and 1 month after brain irradiation (Fig. 8a, b). This is consistent with the permanent cognitive deficits observed after brain irradiation as well as our finding that an M1>M2 signature observed in microglia from normal, aged mice (Fig. 8c). Perhaps more importantly, we identified 3 novel transcriptional modules, each of which demonstrated even higher levels of enrichment in both aging and irradiation than the M1 module. Microglia, like peripheral macrophages, display a wide spectrum of resting and activated phenotypes (Hanisch and Kettenmann 2007; Mosser and Edwards 2008). If a small subset of microglia become M1 polarized after irradiation as previously suggested (Hua et al. 2012), this could explain the presence of M1-like features in our transcriptome due to the presence of a mixed microglial population. Alternatively, a more uniform population of “radiation- or ageing-polarized” microglia may be present that possess some limited features of M1 polarization in addition to other features more specific to the radiated and aged states. Single cell transcriptome studies will be required to address population dynamics in the future. Perhaps the most parsimonious explanation is that the, M1/M2 descriptors, which are based largely on peripheral macrophage experiments, are in fact less relevant to CNS pathology than signatures such as ours, based on microglia acutely isolated from normal and diseased brains without intervening culture.

Extracellular Matrix, Coagulation and Fibrosis

The up-regulation of numerous genes involved in ECM was unexpected and striking. The gene ontology ECM-related genes included connective tissue growth factor (Ctgf), 10 collagens, 3 peptidases including matrix metalloproteinase 2 (Mmp2), a peptidase enhancer, heparinase, and tissue inhibitor of matrix metalloproteinase 3 (Timp3) among others, collectively suggesting changes in both ECM deposition and remodeling. No ECM gene sets were upregulated in publically available M1 or M2 gene arrays (Dirscherl et al. 2010; Mullican et al. 2011), though altered expression of microglial integrins has been reported in response to cytokines and ECM *in vitro* (Milner 2009). LPS administration is used to define the M1 response, but all published gene expression data to date are from macrophages or microglia after *in vitro* LPS administration. Indeed, no data yet exist for freshly isolated microglia from animals treated with LPS *in vivo* to evaluate ECM-changes in microglia responding to LPS in their native environment. Nevertheless, review of published genome-wide expression studies revealed no report of differential expression of ECM gene sets in

freshly isolated microglia from ALS spinal cord, aging retina or cortex, a mouse cuprizone demyelination model, nor in amoeboid or ramified microglia (Chiu et al. 2013; Ma et al. 2013; Olah et al. 2012; Orre et al. 2014; Parakalan et al. 2012). ECM changes were significantly represented in the aged microglia from Hickman et al, as re-analyzed herein (figure 8; Sup Tab 20), however, attention in the prior study only to the microglial sensome precluded identification of these altered ECM genes (Hickman et al. 2013). As such our re-analysis of this previously published RNA-seq data provides important novel insights into the microglial aging process. More recently, a module containing ECM-related genes was also found to be altered in association with late onset Alzheimer's disease using WGNCA (Zhang et al. 2013a).

Microglia and ECM components have both been implicated in synaptic regulation (Dityatev et al. 2010; Lively and Schlichter 2013). GSEA did not reveal enrichment for phagocytosis-related gene sets, however, microglia-mediated ECM changes could modulate synapse formation, strength and astrocyte-mediated elimination (Chung and Barres 2012). Indeed, certain perineuronal net components including *bcn* and *hapln3* were among the ECM genes in the green module for irradiated microglia; perineuronal nets have been implicated in synapse stability and inhibition of plasticity in critical developmental periods (Bavelier et al. 2010). Finally, ECM components can also store and regulate numerous signaling molecules, modulate stem cell homeostasis in diverse niches (Bonneh-Barkay and Wiley 2009; Watt and Huck 2013), and alter axon and myelin regeneration (Lau et al. 2012). Thus, several plausible mechanisms could invoke ECM changes in the radiation-induced cognitive decline seen after irradiation. Lee et al. (2012b) showed that MMP-2, MMP-9, and TIMP-1 are significantly increased within 24 hours following cranial irradiation in mice, leading to a MMP2-dependent decrease in collagen IV in vascular basement membranes. Our results build on these findings by identifying microglia as a persistent source of upregulated MMP2 at both 24h and 1 month after irradiation, while additionally identifying at least 48 other differentially expressed GO-annotated ECM-related genes that altered at 1-month time-point. As with most GO categories, the annotation of relevant genes is not comprehensive, and multiple additional ECM-related genes may also be among those differentially expressed.

Coagulation-related gene sets were also significantly upregulated by GSEA. Components of coagulation pathways, once thought to be relevant only in the acute tissue injury phase, have now been shown to foster a chronic inflammatory state leading to fibrosis in lung, liver, kidney and heart, among other organs (Mercer and Chambers 2013). This correlation is provocative in light of the progressive changes occurring after irradiation, including thickening and hyalinization of irradiated microvasculature (Baker and Krochak 1989). Radiation is known to cause direct damage to ECM components (Aarnoudse and Lamberts 1971). Thus, one could speculate that the ECM changes in microglia are merely reparative. However, the long-term persistence and the progressive accumulation of pathological changes raises the question of whether microglial-induced changes in the ECM may mirror processes of chronic fibrosis after injury in other organs. In fact, IPA canonical pathway analysis showed that the irradiated microglia signature is most significantly enriched for the hepatic fibrosis / hepatic stellate cell activation pathway (Fig. 5a). The potential contributions of ECM-remodeling enzymes to chronic white matter degeneration (Greene-

Schloesser and Robbins 2012; Szerlip et al. 2011) and reportedly increased glioma cell migration following irradiation (Kesanakurti et al. 2013; Kil et al. 2012; Shankar et al. 2013) also warrant further investigation.

Pathway Analysis and Implications for Therapy

Several therapeutic targets have been investigated to ameliorate radiation-induced cognitive deficits, including non-steroidal anti-inflammatory drugs (NSAIDs), peroxisomal proliferator-activated receptor gamma (PPAR γ) agonists, fenofibrate, angiotensin-converting-enzyme (ACE) inhibitors, tannins, lithium chloride, a GLP-1 analogue, tamoxifen and novel small molecules specifically targeting microglia (Dong et al. 2010; Huo et al. 2012; Jenrow et al. 2013; Lee et al. 2012a; Liu et al. 2010; Monje et al. 2003; Parthasarathy and Holscher 2013; Ramanan et al. 2009; Schnegg et al. 2013; Zhao et al. 2007). One study using pioglitazone, a PPAR γ agonist, improved memory function in irradiated rats (Zhao et al. 2007) and is being evaluated in a phase 1 clinical trial (NCT01151670). As such, it is of interest that CEBPA, which was downregulated following radiation, was a major node in the most significant IPA network (Fig. 5) since CEBPA expression may be upregulated by PPAR γ agonists (Zhao et al. 2013). Of note, using IPA analysis, the most significant predicted upstream regulator of the changes induced by irradiation was LPS. PPAR γ agonists ameliorated cognitive deficits and neurogenesis in LPS-treated mice (Ormerod et al. 2013). As such disease-specific microglial transcriptome data may facilitate the identification of appropriate therapeutic agents for neuroinflammatory diseases. Nevertheless, caution is needed in interpretation of microglial transcriptional changes as potential targets for therapy. Use of minocycline which inhibits microglial activation, actually worsened outcomes in a clinical trial of ALS (Gordon et al. 2007) and Hickman et al recently identified upregulation of neuroprotective pathways in aged microglia (Hickman et al. 2013). Additional work will be required to elucidate which irradiation-induced microglial changes represent appropriate responses to mitigate further injury, and what changes could be therapeutically targeted. Finally, extrapolating conclusions from murine inflammatory signaling to human states is not straight forward. (Seok et al. 2013; Takao and Miyakawa 2014). Thus, once transcriptome data for acutely isolated human microglia are available, the extent to which these diverge from the murine data described herein will be a matter of critical importance.

Gene Set Enrichment Analysis of Aged Microglia

Since altered microglial activity is implicated in aging (Wong 2013), we asked if the irradiated microglia transcriptome or one of its functional modules is enriched in microglia from aged brain. Indeed, analysis of the transcriptome of aged murine microglia using GSEA demonstrated that our signature of microglial genes significantly upregulated 1 month after irradiation was the #1 most highly enriched gene set of from among >10,000 curated gene signatures, with strong enrichment of green, pink and red modules (Fig. 8). As such, shared mechanisms likely mediate the pathogenesis of cognitive decline in both normal aging and cranial irradiation and suggest that brain irradiation may induce a premature cognitive aging phenotype. Indeed, normal aging shares many clinical characteristics with the late effects of cranial radiation therapy, such as memory loss, decreased information processing speed, and learning deficits (Yankner et al. 2008).

Moreover, Memantine, routinely prescribed for Alzheimer's disease—the most strongly age-related neurodegenerative disease—recently became the first drug to show cognitive benefit in adult patients following whole brain irradiation (Brown et al. 2013). Our recent meta-analysis of shared transcriptional changes across human neurodegenerative diseases identified substantial overlap with genes altered with normal aging (Li et al. 2014). As such, insights gained into the pathogenesis of radiation and aging-related cognitive changes may also prove relevant to neurodegenerative disease.

Limitations

Although our data provide novel insights into irradiated microglia, several limitations to our irradiated microglial transcriptome should be considered: (i) Our present analysis is limited to murine microglia. To date, no gene expression profiles are available for human irradiated brain, nor are comprehensive transcriptome data available for freshly isolated human microglia in any pathological state. Future additions to the currently limited inventory of transcriptome data will vastly expand the potential for informative bioinformatics analysis. (ii) Analysis of isolated microglia excluded consideration of other interacting cell types such as astrocytes, oligodendrocytes, neurons and endothelium. (iii) Gene expression changes in the hippocampus, selected for its importance in radiation-induced cognitive deficits, may differ from those in other brain regions after irradiation (Hellstrom et al. 2011; Hua et al. 2012) (Lee et al. 2013; Mizumatsu et al. 2003). However, the close similarity between the signatures of irradiated hippocampal microglia and aged microglia from whole brains (Hickman et al. 2013) suggest that microglial responses are likely conserved in multiple brain regions. (iv) Our results after a single 10 Gy dose—common among rodent studies to avoid confounders of blood brain barrier disruption and radionecrosis—may differ from findings after fractionated radiation or higher doses. (v) Analysis of bulk populations of microglia ignores the potential diversity of individual microglial subtypes within the bulk population. (vi) As an exclusively transcriptional analysis, changes in corresponding protein levels for the genes identified cannot be directly inferred. As such, additional work will be required to build a more comprehensive understanding of radiation-induced CNS changes in the future. We hope that this transcriptome of irradiated microglia will prove a useful resource for research into radiation-induced brain injury, aging, and other neuroinflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Magdalena Bazalova for assistance with irradiator calibration and TLD readings, Dena Leeman and Patty Lovelace for assistance with optimizing our FACS protocols, along with Kambiz Ansari and Dominic Rodriguez for technical assistance. We thank Mariko Bennett for helpful discussions. Funding was provided by NIH R01 MH071472 (TDP), California Institute of Regenerative Medicine Clinical Research Fellowship (TCB), Stanford Medical Scholars Research Fellowships (MDL, SK) and an American Brain Tumor Association Medical Student Summer Fellowship, supported by Dropping the Puck on Cancer at Indiana University (MDL).

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Main Points

The irradiated mouse microglia transcriptome is unique, featuring changes in extracellular matrix, inflammation and mitochondria-related genes. Strong enrichment for irradiation-induced changes is observed in the aging microglia transcriptome.

Figure 1. Schematic of experimental design to generate the irradiated microglia gene signature
 Each mouse figure represents 3 replicates. Fluorescence-activated cell sorting (FACS) plot is representative of gating for CD11b+ cells, following propidium iodide-positive cell exclusion of dead cells. R, R/Bioconductor; GSEA, Gene Set Enrichment Analysis; WGCNA, Weighted Gene Co-Expression Correlation Network Analysis; IPA, Ingenuity Pathway Analysis.

Figure 2. C57BL/6 and Balb/c mice demonstrate a similar response to radiation

(A) Venn diagrams show overlapping significantly differentially expressed genes, on a background of 10,314 genes tested post-filtering. P-value $< 2.2 \times 10^{-16}$ calculated using Fisher's Exact Test for pair-wise comparisons. (B) Heatmap of the log fold change of genes that respond significantly differently to radiation between C57BL/6 and Balb/c mice. (C) Principal components analysis. The first principal component divides the samples by mouse background (C57BL/6 vs. Balb/c), while the second principal component divides the samples by radiation status (sham irradiation vs. 10 Gy irradiation). (D) Heatmap of the log fold change of the top 1000 highest interquartile range genes illustrates that similar groups of genes are differentially expressed in response to radiation in both mouse strains.

Figure 3. Network of gene sets enriched for in the irradiated microglia gene signature
 GSEA for gene ontology (GO) terms (A) and pathway gene sets (KEGG, Biocarta, Reactome) (B). Blue nodes represent significantly enriched gene sets (FDR q-value < 0.05). The size of the node is proportional to the number of genes in the gene set, and a darker node color denotes increased significance of enrichment. Gene sets with > 50% of genes overlapping another gene set are connected by green edges. The edge size is proportional to the number of genes shared between two gene sets; thus, related gene sets cluster together. See fully annotated network and table of significantly enriched gene sets in supplementary materials (Sup. Fig. 5 and Tables 9, 10). ECM, extracellular matrix.

Figure 4. Analysis of irradiated microglial polarity

Hippocampal microglia at 1 month post-cranial radiation demonstrate enriched expression of genes associated with human M1 macrophage polarization. (A) GSEA shows that irradiated microglia, in both strains separately and when combined, are enriched for an M1 macrophage-associated gene modules gene set ($p < 0.001$), but not the human M2 gene set ($p > 0.05$), both adapted from Xue et al. (2014). (B) Heat map analysis for genes frequently employed in the literature to phenotype macrophages, reveals no selective pattern of expression of M1 or M2 markers).

Figure 5. Ingenuity Pathway Analysis (IPA) identifies pathways and hub genes in irradiated microglial gene dysregulation

(A) Top 10 canonical pathways in IPA. Blue bars indicate $-\log(p\text{-value})$, while yellow points denote the ratio of genes from the irradiated microglia gene signature in the gene set of interest to the total number of genes in the gene set of interest. (B) The highest ranked network generated from the irradiated microglia signature consisting of genes associated with the IPA categories DNA replication, recombination, and repair, cardiovascular disease and congenital heart anomaly. Red = upregulated, Green = downregulated, Solid line = direct interaction, Broken line = indirect interaction. Refer to IPA website for complete IPA networks legend.

Figure 6. Weighted gene co-expression network analysis (WGCNA) divides the irradiated microglia gene signature into three functional groups

(A) Heat map shows the correlation of gene co-expression modules (color names) with radiation status and mouse strain. Numbers overlaying heat map denote Pearson correlation coefficients (top number) and p-values (lower number in brackets). Positive (negative) correlations indicate correlation with radiation treatment (sham treatment) or Balb/c (C57BL/6) background. (B) Histogram of the number of genes from the irradiated microglia gene signature in WGCNA gene co-expression modules. (C–E) EnrichmentMap representation of DAVID analysis for GO enrichment of genes in select modules (Green Module (C), Pink Module (D), and Red Module (E)). Blue nodes represent gene sets that are enriched in the specific module (Benjamini-Hochberg corrected p-value < 0.05). The size of the node is proportional to the number of module genes in the gene set, and the darker the node the greater the significance of enrichment. Gene sets with > 50% of genes overlapping another gene set are connected by green edges. The edge size is proportional to the number of genes shared between two gene sets; thus, related gene sets cluster together. See fully annotated networks and table of significantly enriched gene sets in supplementary materials (Sup. Fig. 8 and Tables 14, 15, 16). ECM, extracellular matrix.

Figure 7. Heat map for significantly differentially expressed genes (the irradiated microglia gene signature)

Bars to the left annotate genes included within WCGNA-determined gene modules significantly correlated with radiation treatment. Genes are ranked from highest to lowest fold change, top to bottom and left to right. Log2(FC), log2 transformed fold change.

Figure 8. Gene set enrichment analysis (GSEA) for custom gene sets in irradiated and aging microglia

(A) 24 hours following radiation, (B) 1 month following radiation, and (C) normal aging microglia (24 months versus 5 months). Custom gene sets correspond to the upregulated and downregulated components of the irradiated microglia gene signature (Rad Resp Up and Rad Resp Down), the significantly differentially expressed components of select modules (Green Module, Pink Module, Red Module), and human M1 and M2-polarized macrophage gene sets from the literature (M1 Modules and M2 Modules). Normalized enrichment scores provide a measure of the degree of enrichment, but are not directly comparable between experiments. The blue line indicates the approximate normalized significance threshold; Gene sets with NES above ~1.4 were significant ($FDR < 0.05$). NES values above ~1.9 were highly significant ($FDR < 0.001$)