**Transcript Profiles of Microglia Associated with Chronic Active MS Lesions**

**Introduction**

Microglia, the resident innate immune cells of the central nervous system, form a network of cells that covers the entire brain parenchyma. Microglia have small spindle-shaped cell bodies and extend symmetrically oriented processes that are constantly monitoring their microenvironment. Processes of neighboring microglia rarely overlap, so each microglia cell is responsible for monitoring the small microenvironment where they reside. During brain development, microglia remove dead cells and prune synapses by a process termed synaptic stripping. In normal conditions, microglia cell bodies are stationary and rarely divide. During disease states, however, microglia become activated, proliferate, migrate to sites of pathology and remove cellular debris. Activated parenchymal microglia play a role in chronic brain pathology as environmental mediators of oxidative stress in most neurodegenerative diseases. Microglia senesce plays a role in chronic brain diseases by loss of their protective role {17}.

Dynamic and diverse microglial responses are found in the immune-mediated demyelinating disease multiple sclerosis (MS). GWAS studies? The microglia response to demyelination varies based upon lesion location, mechanism of demyelination and age of the lesion. White matter demyelination is mediated by infiltrating immune cells. Monocytes are the major peripheral immune cell that enters the acute white matter (WM) lesion. Microglia within the acute WM lesion become phagocytes and cannot be distinguished from phagocytes derived from peripheral monocytes. The life span and utility of microglia is shortened when they turn into phagocytes. They do not re-differentiate into microglia and either degenerate or enter the circulation where they are targeted to the liver and destroyed. Microglia eventually repopulate chronic WM lesions which are divided into chronic inactive or chronic active lesions based upon the accumulation of microglia at the myelinated lesion border of chronic active, but not chronic inactive lesions. Brain imaging studies have reported slow expansion of chronic MS lesions that have an iron-enhancing ring at their border. Microglia are iron sequestering and may participate in lesion expansion of chronic active lesions and progression of neurological disability in MS. Pathological and molecular characterization of microglia that occupy chronic active lesions with iron ring enhancement has been a challenge. Iron enhancing lesions are detected in X% of MS brains and X% of MS brains have 1 iron enhancing lesion. The remaining X% have multiple (x-Y) iron ring enhancing lesions.

The mechanism of demyelination and microglial response in subpial cortical lesions differs significantly from that in white matter lesions. Subpial cortical lesions are abundant, often restricted to cortical layers I-IV, transverse several gyri and contain few peripheral immune cells. On occasion, they can involve all 6 cortical layers, but they rarely, if ever, invade subcortical white matter. Phagocytic macrophages are not a prominent feature of subpial lesions. Subpial lesion contain activated microglia which occasionally form a microglial line at the border of the subpial lesion. These microglial lines have not been reported to be iron-enriched when analyzed by MRI. There is a predilection for subpial lesions in cortical areas with deep sulci and speculation that meningeal inflammation and possibly B cell follicles in the CSF space of deep sulci play a pathogenic role in inducing subpial demyelination. With the exception of loss of myelin and microglial activation, subpial lesions lack many of the pathological signatures of white matter lesions. They do not have breakdown of the blood-brain-barrier, infiltration of immune cells, perivascular cuffs, astrogliosis, loss of oligodendrocyte progenitor cells, or complement activation.

CD11b-positive microglial transcriptional profiles have been compared in myelinated gray matter (GM) and myelinated white matter (WM) from non-neurological control donors and MS patients {van der Poel et al, Nat Communications, 2019}. While the majority of microglial transcripts were similar across these subgroups identifying a homeostatic microglial transcript profile, there were differences. Type-1 interferon genes were increased in control GM microglia and NF-Kb genes were increased in control WM microglia. Microglia from MS WM showed increased expression of lipid metabolism transcripts, while microglia from MS GM showed increased expression of transcripts associated with glycolysis and iron homeostasis. In a study of CD45-positive cells isolated from human cortex, snRNA-seq identified 7 microglial cell clusters and 3 of these clusters appeared to be enriched in MS brains {Masuda et al, Nature, 2019}. snRNA-seq of cerebral cortex identified a phagocytic microglial subgroup and raised the possibility that microglia isolated from MS cortex ingest myelin protein transcripts and translocate them to perinuclear structures or the nucleus {Schrimer et al, Nature, 2019}.

Establishment of microglial genotypes in discrete locations in MS brains is a challenge (Gerrits at al, 2020). Microglia yield is relatively low in single cell or single cell nuclei approaches. To overcome these limitations, the present study characterizes the transcriptional profile of laser captured microglia using RNA-seq. Specifically, we focus on gene transcripts captured from the microglia rim of chronic active white matter lesions and compare these transcripts to transcripts from microglia lines at the border of subpial cortical lesions. Our results show the value of the approach by hierarchical clustering of significantly altered transcripts and the identification of DEG’s in each location.

**Materials and Methods**

*Tissue Selection for Laser Capture.* This research was approved by the Cleveland Clinic Institutional Review Board. Tissue donation from patients with MS was obtained with consent from the patient or next of kin. We compared RNA-seq profiles of laser captured microglial located at the borders of white matter lesions (n=5) and subpial cortical lesions (n=5). These regions of interest were identified on 10um-thick cryostat sections of frozen blocks obtained from hemispheric cm-thick brain slices obtained from the rapid autopsy program at the Cleveland Clinic (Jove paper). WM and subpial cortical lesions were identified by myelin staining of 10 um-thick cryosections. Microglial lines at the border of WM and subpial lesions were identified by MHC Class II staining. Disease course and demographic characteristics of the patient cohort are listed in Supplemental Fig 1.

*Laser capture of microglia and RNA isolation.* After lesion and immune cell status was confirmed, frozen tissue blocks were sectioned (8-10 µm) and a modified immuno “quickstain” was performed with CD68 antibody. The “quickstain” is a modified immunotaining protocol with short incubation times (<5min) in RNase free buffers. Individual or clusters CD68 immuno-positive cells (≥4000) from WM line (n=5) and GM line (n=5) were collected via infrared laser capture (LCM) onto caps using Arcturus XT (figure 2). Collected cells were lysed and RNA was isolated in buffer RLT (Qiagen RNeasy micro kit). RNA quality was measured after isolation from LCM collected cells with a Bioanalyzer (Agilent Technologies)(figure 2E).

*Library Preparation and Sequencing.* RNA quality evaluation and quantification were performed on Advanced Analytical’s Fragment Analyzer and its High Sense RNA kit. Total RNA was normalized to 100 pico grams prior to oligo-dT capture and cDNA synthesis with Takara’s SMART-Seq v4. The resulting cDNA was assessed on the Fragment Analyzer with the High Sense Large Fragment kit and quantified using a Life Technologies’ Qubit 3.0 fluorometer. Libraries were generated using Illumina’s Nextera XT DNA Library Prep kit. Medium depth sequencing (>30 million reads per sample) was performed with an Illumina HiSeq 2500 on a Rapid Run v2, 125 base pairs, Paired End run.

*RNA-Seq Analysis.* We prepared 10 RNA libraries in total (5 libraries per group) On average, we obtained about 11 million reads (3-23 million) per sample, and the average mapping of 76.43% (49-92 %) to the GRCh38 reference genome using HISAT2 (reference1) after trimming of adapters through cutadapt (reference2). Next, we employed DESeq2 (reference 3) to normalized raw counts with median of ratios methods after filtering >10 reads per gene and to determine the differentially expressed genes and pathways from comparisons. Plots and heatmaps are obtained in R/Bioconductor (R version 4.1.1). All data is available in GEO (accession # GSEXXXXX)

*Functional and Pathway Analysis.* Over-representation analysis of differentially expressed genes by DESeq2 was performed using Enrichr (reference 4) to identify enriched Gene Ontology (GO) (reference 5) categories and/or pathways. The predefined pathways we used are compiled from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (reference 6) and Hallmark (reference 7). Hallmark contains 50 gene signatures from over 4,000 original overlapping gene sets from v4.0 MSigDB collections and KEGG contains 186 gene signatures in c2.cp.kegg.v7.4 (MSigDB). In addition, to identify enriched brain cell types in each group, we used Gene Set Enrichment Analysis (GSEA) (reference 8, Subramanian et al., 2005) with a combination of BrainRNAseq dataset (brainrnaseq.org, reference 9) and our house dataset as genesets and normalized count values as expression dataset. Enriched genesets were identified by signal-to-noise method with 1000 permutations. Gene sets with normalized enrichment scores (NES) > 1.5 or < -1.5 were considered significant.

*Credentialing.* To validate the cellular specificity and location of translational products of DEGs, immunocytochemistry was performed on 30-um thick free floating sections obtained from brain slices fixed with 4% paraformaldehyde. We focused on translational products of DEGs that were integral to functional pathways and that showed a significant (p < 0.01) change in expression levels. Antibodies used in these studies are described in Supplemental Figure X.

*Iron staining.* The diaminobenzidine (DAB)-enhanced Turnbull staining protocol was applied for detection of iron. After washing free floating tissue was immersed in aqueous ammoniumsulfide solution [2% in deionized water (d.w.)] for 90 minutes This process followed by a 30-minute incubation with aqueous solution containing 5% potassium ferricyanide and 0.5% hydrochloric acid (HCl). After washing steps with dH2O, sections were incubated in methanol containing 0.01 M sodium azide and 0.3% hydrogen peroxide for 60 minutes for blocking of endogenous peroxidase. Next, sections were washed with 0.1 M Sorrenson’s buffer. Iron staining was amplified by a solution containing 0.025% DAB (Sigma Aldrich) and 0.005% hydrogen peroxide in a 0.1 M phosphate buffer for 5-10 minutes (for proper color developing). The reaction was stopped by rinsing the sections in tap water and mount in glycerol as regular mounting protocol (figure 3D).

**Results**

*Microglial Lines in Postmortem MS Brains.* We characterized postmortem MS lesions with PLP and MHC Class II antibodies. PLP antibodies defines the MS lesion and MHC Class II antibodies identifies microglia/monocyte associations with the lesion. Chronic active WM MS lesions are characterized by a marked accumulation of microglia at the lesion border (Fig. 1). These microglia rings can totally (Fig. 1) or partially (see Fig. 2) line the lesion border. The content of microglia in chronic active lesions varies, but is usually less than found in normal appearing white matter. Chronic active lesions, therefore, are characterized by a microglial ring and low density of lesional microglia.

Cortical lesions are divided into two major subtypes: subpial lesions and leukocortical lesions. Subpial lesions are the most abundant and characterized by a subpial band of demyelination that extend from the pial surface. Most subpial lesions stop at cortical layer IV (Fig. 1) and some have microglial lines (Fig. 1). These microglial lines are much thinner than the microglial lines of chronic active white matter lesions. When present they tend to line the entire length of individual subpial lesions which can occupy an entire cortical gyrus. Monocyte/macrophages are not a prominent feature of the subpial lesion area. In subpial lesions that demyelinate all six cortical layers the microglial line at the grey matter/white matter junction can be very prominent (Fig 1). Leukocortical lesions demyelinate both subcortical white matter and lower layers of the cerebral cortex. Leukocortical lesions can be staged based upon the density and location of MHC Class II-positive cells. Active leukocortical lesions are identified by an abundance of monocytes/macrophages in the subcortical white matter { }. Cortical portions of active leukocortical lesions have significantly less macrophages/monocytes than their white matter counterpart, but more than myelinated cortex or subpial lesions { }. White matter, but not cortical portions of leukocortical lesions can Gad-enhance in living MS patients and cortical portions of active leukocortical lesions rarely contain perivascular cuffs of immune cells. It is most likely that leukocortical lesions originate in the subcortical white matter and invade the cerebral cortex. Leukocortical lesions can also contain a microglial line (Fig. 1). Microglial lines are thicker in the white matter portion compare to the cortical portion of leukocortical lesions (Fig1) and the gray matter line is most often more prominent closer to the white matter lesion (Fig 1).

*Laser capture of microglia and RNS isolation*. Microglia were laser captured from frozen MS tissue blocks as described in methods. White matter lesions were identified macroscopically on cm-thick frozen slices, removed, sectioned at a thickness of 10ums and stained with PLP and MHC class II antibodies. Chronic active lesions were identified by a MHC Class II-positive microglial line at the lesion border (Figs. 2a and b). Subpial cortical lesion with microglial lines were identified by staining cryosections from cortical blocks with the same antibodies. Once microglial lines were identified, additional 10um-thick cryosections were stained for CD68 antibodies using a rapid immunostaining method (< 5 minutes). Stained sections were placed in the laser capture microscope and a minimum of 4,000 cells were collected from 5 white matter lesion microglial lines and 5 subpial lesion microglial lines and immediately placed in RNA extraction buffer (Tony please embellish if necessary). Figs. 2c and d shows a chronic active white matter lesion and the microglia collected by LCM.

RNA integrity number (RIN) was determined for each sample using an Agilent Bioanalyzer (Fig. 2e) and samples with RINs > 5 we used for RNA seg. The enrichment of microglial genes was obtained from Gene Set Enrichment Analysis (GSEA) (reference 8, Subramanian et al., 2005) and several gene sets as described in methods. We compared WM and GM microglial line cell-type-specific gene enrichment with RNA samples from normal appearing white matter (Fig. 2f). Gene sets with normalized enrichment scores (NES) > 1.5 or < -1.5 were considered significant. Microglial-specific transcripts were highly enriched in WM and GM microglial lines compared to NAWM (Fig. f, left panels). In contrast oligodendrocyte specific transcripts were highly enriched in NAWM, but not in WM or GM microglial lines (Fig. 2f, right panels).

*RNA-seq of white matter line vs gray matter line.* We used RNA sequencing to compare gene transcripts in laser captured microglia from WM (n=5) and GM (n=5) lesion lines. One hundred and eighty-six gene transcripts were significantly altered (FDR <0.05) as shown in the heatmap (Fig. 3a). The volcano blot (Fig. 3b) 176 of these differentially expressed genes (DEGs) were increased in WM lines and 111 were enriched in GM lines. Using the brain cell-specific *RNA-seq* database (brainrnaseq.org, reference 9), 11 of these DEGs were identified as microglia specific or microglia enriched which is defined by pval < 0.05 and FC > 2 comparison with other cell types. Using Gene Ontology (GO) analyses three pathways were significantly increased in white matter lines when compared to gray matter lines. These include iron homeostasis (GO:0006879; p> 0.0015), cytokine signaling (GO:0019221; p>0.0041)

Of the 111 DEGS enriched in GM microglia lines, x were specific for or enriched in microglia based upon the brain cell-specific *RNA-seq* database ( ). Using Gene Ontology (GO) analyses five pathways were significantly increased in gray matter microglia lines when compared to white matter microglia lines. These include protein folding (GO:0034629; p< 0.0002), heat shock protein response (GO; 0034620; p< 0.0001) and conical Wnt signalling (Go:0060070; p> 0.0007).

*Credentialing.* To investigate the validity of laser capture gene profiles, we performed a series of validation studies to determine whether translational products encoded by differentially expressed microglial transcripts where enriched in appropriate cells and locations. We focus on WM line microglia and the roles of iron homeostasis and NFkb signaling. Both pathways have been implicated in white matter lesion expansion and disability progression in progressive MS { }.

Four iron related transcripts were significantly increased in WM lines: FXN, SLC11A1, TFRC and HAMP (Fig. 4a and b). We first investigated whether iron was detectable in WM and GM microglial lines. Iron was present in microglia associated with microglial lines of white matter lesions, but not in microglia associated with GM lines (Fig. 4c). Should we expand this?. We also credentialed TFRC and HAMP. TFRC encodes the transferrin receptor C and play a role in …. Transferrin receptor C was detected in microglia of WM lines, but not microglia of GM lines (Fig. 4d). Hamp encodes hepcidin an iron sequestering protein that degrades ferroprotein (FPN). Need another sentence. We compared the location of hepcidin in white and gray matter lesions that contained microglial lines. Staining patterns supported gene profiling studies by associating increased hepicidin staining in WM lines compared to GM lines (Fig. 4d). HAMP was also highly enriched in large, round, phagocytic macrophages located within, but concentrated at the edge of some chronic active WM lesions. We examined X chronic active lesion and found variable densities of these lesional helicidin-positive macrophages in Y/X chronic active lesions. These hepicidin-positive phagocytes were positive for MHC Class II and CD 68, but negative for the microglial cell marker Iba1 (Fig. 5), suggesting that they may be of vascular origin. Overall, these observation are consistent with reports of iron enhancing rings surrounding WM lesions, but not GM lesions in living patients using T2 star MRI sequences { }. The role of hepiciden-positive phagocytes in some chronic active white matter lesions remains to be determined.

Bruton’s Tyrosine Kinase (BTK) is expressed by B cells and microglia, involved in NF-kappa *B* signaling and currently a therapeutic target in MS clinical trials. Upon KEGG pathway analysis, the NF kappa *B* signaling pathway was significantly increased in WM line microglia (Fig. 5a). Transcripts included BTK, PLAU, STK, TRM25 and GxDD45B. Laser capture studies found a 4 fold increase in BTK transcripts in WM line when compared to GM line (Fig. 6a). Immunocytochemical studies confirmed this and provided additional information (Fig. ). Tony- I will talk to Sanofi and see where their paper stands and see what BTK data we can add to our paper. They will be co-authors

**Discussion**

Major functions of microglia are to help maintain brain homeostasis. In normal conditions, this homeostasis is restricted to and influenced by the microdomain in which they reside. The distribution, location and function of microglia is altered by brain pathology. One of the most dramatic microglial responses is their accumulation at the border of demyelinated lesions in MS brains. We report that transcript profiles of microglia that line the border of cerebral WM and subpial cortical lesions differ. The WM microglial line is a therapeutic target in MS. We identify up regulation of iron homeostasis and NF kappa B signaling transcripts in WM, but mot GM line microglia.

The technology of single-cell RNA sequencing (scRNA seq) has now become the dominant technology enabling to profile the transcriptome of each individual cell in the tissue sample while bulk RNAseq averages gene expression across all the cells in the sample. Although scRNA seq shows several benefits over bulk RNAseq Postmortem brain tissue has still limitation in cell preparation with low sensitivity. snRNA is a good replacement of scRNA in brain research however a recent publication warned taht snRNA is not suitable for detection of microglia activation in humans (Thrupp et al., Cell reports, 2020) and successful studies also cannot avoid low depth problems in single cell or single nuclei sequencing studies.

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