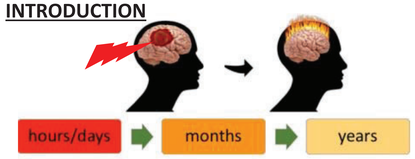
Lysosomal Lipid Accumulation in Mononuclear Phagocytes Impairs Autophagy and Contributes to Inflammation after Traumatic Brain Injury

Amir Mehrabani-Tabari[1], Nivedita Hegdekar[1], Chinmoy Sarkar[1], Jace Jones[2], Maureen Kane[2], and Marta Lipinski[1]

University of Maryland School of Medicine Department of Anesthesiology[1], University of Maryland School of Pharmacy Department of Pharmaceutical Sciences[2]

INTRODUCTION



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Prolonged inflammation after TBI

Inflammation resolution does not complete after TBI and leads to chronic neuroinflammation.

Microglia-mediated neuroinflammation is of special focus among factors contributing to second injury after TBI.

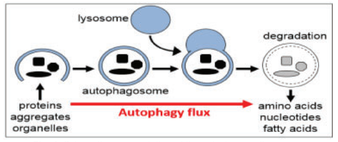

Fig. 1. Schematic representation of the autophagy pathway.

Autophagy impairment in proinflammatory monocytes after TBI

Data from our lab demonstrate that autophagy is inhibited after TBI in parallel with upregulation of proinflammatory markers in mononuclear phagocytes, but the mechanistic explanation behind the autophagy impairment remains unknown.

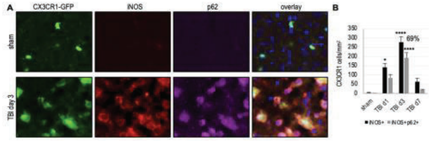

Fig. 2. Immunostaining for autophagy and inflammatory markers in coronal brain sections. Proinflammatory monocytes including resident microglia and infiltrating macrophages demonstrate P62 accumulation as a marker for autophagy inhibition.

Lipid accumulation can lead to inhibition of autophagy

It is shown that infiltrating macrophages phagocytose lipids in atherosclerotic plaques and become proinflammatory foam cells.

In multiple sclerosis is also shown that myelin-laden macrophages are reminiscent of foam cells. Cholesterol rich species like myelin are abundant in TBI lesion. We hypothesized that lipid phagocytosis by monocytes in TBI lesion can cause autophagy inhibition.

MATERIALS AND METHODS

Moderate TBI was induced in C57BL6/J WT mice using controlled cortical impact (CCI) injury model. After injury animals were sacrificed at different time points and their brains were removed and subjected to either coronal cryo-sectioning for immuno-fluorescent staining and mass spectrometry imaging, or peri-lesion cortices were dissected for LC-MS/MS lipidomic analysis, or the ipsi-lateral hemispheres were used to isolate mononuclear phagocytes for either flowcytometry analysis or fluorescent activated cell sorting (FACS) that was submitted for LC-MS/MS lipidomic analysis. For in vitro assays, bone marrow derived macrophages from C57BL6/J WT mice were grown in ether twelve well plates and lysed for western blot or on coverslips in 24 well plates for immunostaining.

RESULTS

Neutral Lipids Accumulate in Perilesional Tissue of the TBI Brain

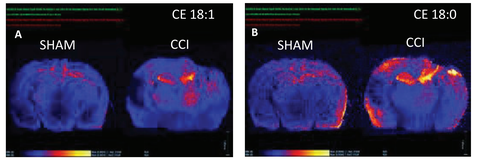

Fig. 3. DESI-MSI and cell-type specific LC-MS/MS data generated from our TBI mouse model. A and **B** Desorption electro-spray ionization – mass spectrometry imaging (DESI-MSI) of CCI day 3 brain sections representing two types of cholesteryl esters (esterified with oleic acid and stearic acid respectively). **DESI-MSI combines two essential techniques, desorption and electrospray ionization. It uses a spray of charged solvent droplets to desorb molecules from the sample surface, which are then ionized for mass spectrometry analysis. C**. Fluorescent activated cell sorting (FACS) purified microglia and macrophages from TBI samples further subjected to lipidomic analysis. CD11b and CD45 were used as surface markers as CD11b being the common marker expressed in both populations and then CD45 subdivided into two clusters, CD45^{int} representing infiltrating monocytes and CD45^{hi} representing microglia. **D.** principal component analysis shows distinguished lipid content in FACS purified microglia versus macrophages **E**. Infiltrating macrophages from TBI day 3 samples show more triglycerides and sphingomyelin accumulation compared to microglia.

Lipid Droplets accumulate in microglia and macrophages after TBI

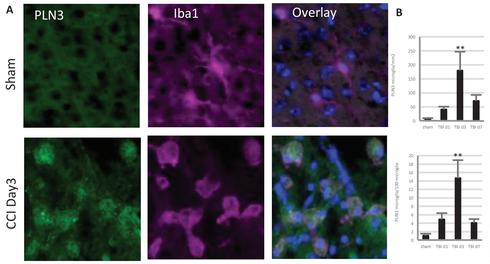

Fig. 4. Expression of lipid droplet marker, Perilipin 3 (PLN3), in ipsilateral cortex of TBI brain. A. Perilipin3 is a ubiquitous lipid droplet coating protein that in this experiment shows elevated accumulation in Iba1 positive cells (monocytes) after CCI that peaks at day 3. Lipid droplet accumulating microglia (LDAM) has been recently identified as a specific phenotype that contribute to more proinflammatory cytokines and ROS production that also show phagocytosis deficiency. **B.** All data are mean ± SEM; n= 3-4/ time point; **p<0.01 vs Sham (One- way ANOVA with Dunnett's post hoc test for multiple comparison).

Neutral Lipid Accumulating Monocytes Demonstrate Autophagy Impairment after TBI

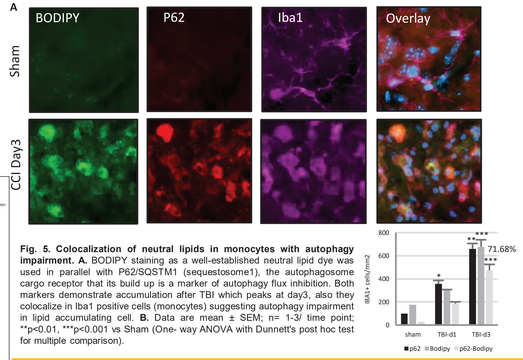

Fig. 5. Colocalization of neutral lipids in monocytes with autophagy impairment. A. BODIPY staining as a well-established neutral lipid dye was used in parallel with P62/SQSTM1 (sequestosome1), the autophagosome cargo receptor that its build up is a marker of autophagy flux inhibition. Both markers demonstrate accumulation after TBI which peaks at day3, also they colocalize in Iba1 positive cells (monocytes) suggesting autophagy impairment in lipid accumulating cell. **B.** Data are mean ± SEM; n= 1-3/ time point; **p<0.01, ***p<0.001 vs Sham (One- way ANOVA with Dunnett's post hoc test for multiple comparison).

Neutral Lipid Accumulating Monocytes Demonstrating Autophagy Impairment also show exacerbated inflammation after TBI

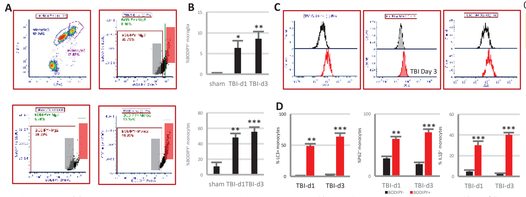

Fig. 6. A. FACS gating approach to divide mononuclear phagocytes (CD11b$^+$) into resident microglia (CD45^{int}) and infiltrating monocytes (CD45^{hi}). Furthermore, each cell type was gated upon BODIPY, so we ended up having cells with high lipid (BODIPY$^+$) versus low lipid (BODIPY$^-$). **B.** Data are mean +/- SEM; n=6; *p<0.05, **p<0.01, and ***p<0.001 vs sham (One- way ANOVA with Dunnett's post hoc test for multiple comparison). **C.** Histograms representing LC3, P62, and IL1β expression in infiltrating monocytes after TBI. **D.** Data are mean +/- SEM; n=6; **p<0.001, ***p<0.0001 vs BODIPY$^-$ at each time point (Welch's t-test).

Neutral lipids accumulate in microglial/macrophage lysosomes after TBI

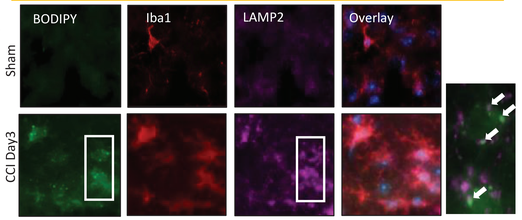

Fig. 7. Co-localization of lipid accumulation with lysosomes. Neutral lipid dye (Bodipy in green) co-localizes with lysosomal membrane marker (LAMP2 in far red) in monocytes (Iba1-positive cells) in TBI lesion. It is known that neutral lipid accumulation in lysosomes can lead to lysosomal dysfunction that eventually inhibits the last step of autophagy, degradation.

Lipidomic Analysis of Lysosomes after TBI Suggests Myelin Accumulation

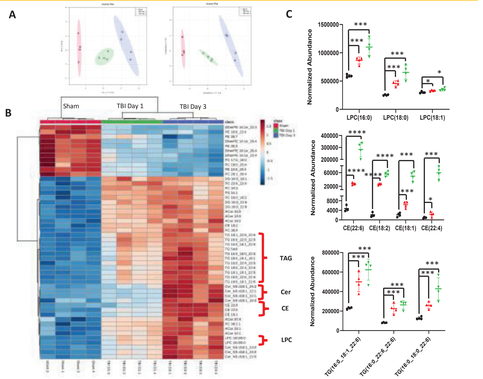

Fig. 8. LC-MS/MS lipid analysis of purified lysosomes isolated from mice cortices comparing sham and CCI-induced TBI. A. PCA and PLS-DA plots. **B**. Hitmap showing details of distinct lipid content of lysosomal samples. **C**. Altered abundances of lysophosphatidylcholines, cholesterol esters, and triacylglycerides demonstrated at 1 and 3 days post-TBI. Data obtained was evaluated by multiple t-test comparisons using the Holm-Sidak method. Statistical significance was determined by +p < 0.05, +p < 0.01, +++p < 0.005, ++++p < 0.0001. Individual data points were reported as mean ± SD.

Myelin Exposure Inhibits Auto-Lysosomal Degradation in BMDMs

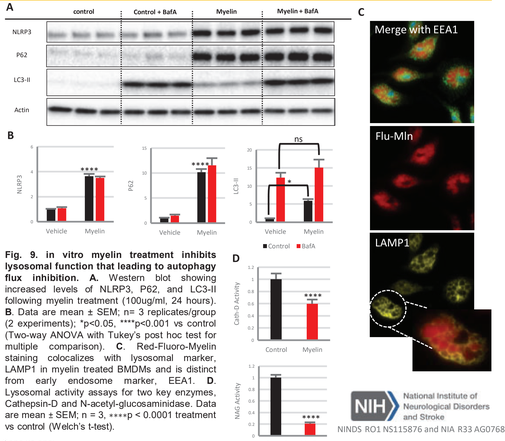

Fig. 9. in vitro myelin treatment inhibits lysosomal function that leading to autophagy flux inhibition. A. Western blot showing increased levels of NLRP3, P62, and LC3-II following myelin treatment (100ug/ml, 24 hours). **B**. Data are mean ± SEM; n= 3 replicates/group (2 experiments); *p<0.05, ****p<0.001 vs control (Two-way ANOVA with Tukey's post hoc test for multiple comparison). **C**. Red-Fluoro-Myelin staining colocalizes with lysosomal marker, LAMP1 in myelin treated BMDMs and is distinct from early endosome marker, EEA1. **D**. Lysosomal activity assays for two key enzymes, Cathepsin-D and N-acetyl-glucosaminidase. Data are mean ± SEM; n = 3, ****p < 0.0001 treatment vs control (Welch's t-test).

Conclusions:

- Neutral lipid accumulates in the lysosomes of mononuclear phagocytes after TBI with the composition suggesting it is myelin.
- Lysosomal accumulation of myelin in macrophages in vitro causes inhibition of auto-lysosomal degradation that exacerbates inflammation.
- This study provides evidence for novel therapeutic targets to improve TBI outcomes focusing on crosstalk between lipid metabolism, autophagy, and inflammation.

Future Directions

- Confirm lysosomal dysfunction in monocytes after TBI in vivo.
- Study the gene expression of the key mediators of lipid metabolism in monocytes focusing on cholesterol handling genes.

NIH National Institute of Neurological Disorders and Stroke

NINDS RO1 NS115876 and NIA R33 AG076858