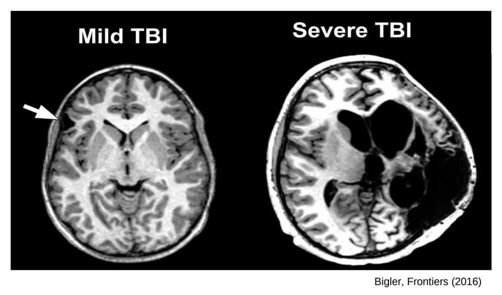
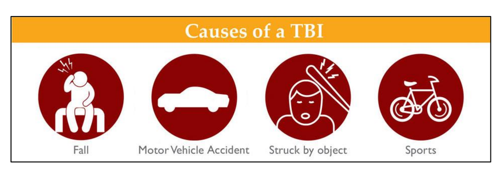
The Role of LC3-associated Phagocytosis in Traumatic Brain Injury Mice Model

Sagarina Thapa, Amir Mehrabani Tabari, Nivedita Hegdekar, Chinmoy Sarkar, and Marta M. Lipinski
Shock, Trauma and Anesthesiology Research (STAR) Center, Department of Anesthesiology
University of Maryland School of Medicine, Baltimore, MD

UNIVERSITY of MARYLAND SCHOOL OF MEDICINE

Introduction





Traumatic Brain Injury (TBI) is a leading cause of death and disability in the US. It occurs when a strong external force impacts the brain within its skull. The secondary stage of TBI involves cellular and metabolic changes resulting from the disruption of the blood brain barrier and cerebral edema, contributing to neuroinflammation and further neurodegeneration. Both primary and secondary injuries result in neuronal cell death and release of endogenous Damage Associated Molecular Patterns (DAMPs).

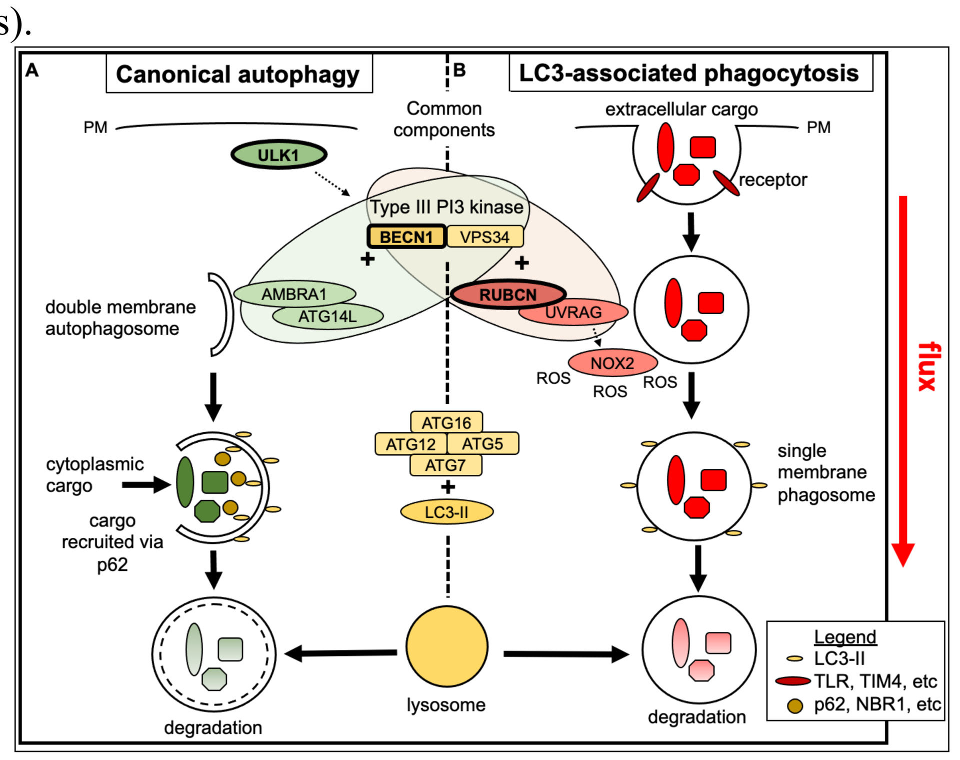
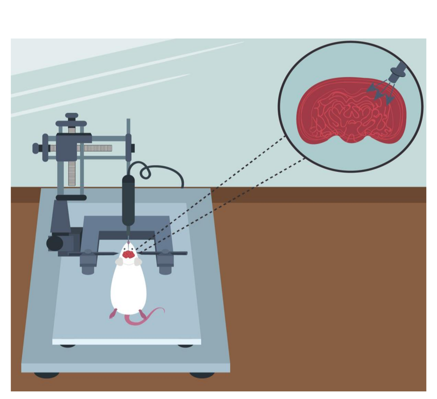


Figure 1. Overview of the canonical autophagy (A) and LC3-associated phagocytosis (B) pathways.

Macroautophagy (canonical autophagy) and **LC3-associated phagocytosis (LAP)** are lysosome-dependent catabolic processes involved in maintaining cellular homeostasis. *Becn1* regulates both canonical autophagy and LAP (also termed as non-canonical autophagy), each of which has been implicated in the regulation of inflammation. While LAP shares some components, such as Beclin1 and LC3, with autophagy, it uniquely requires presence of the type III PI3 kinase component, Rubicon, and the phagocytic mediator NOX2. LAP is involved in phagocytosis of extracellular cargoes such as dead/dying cells and DAMPs, which are abundant after TBI.

Methods

❑ *In vivo*



- *C57Bl/6* mice expressing *Lyz2-cre/Becn1-flox* (*Becn1 cKO*), *GFP-LC3*, and Rubicon knock-out (*Rubcn KO*) were used. TBI was induced on mice via Controlled Cortical Impact (CCI) injury device with an impact velocity of 4 m/s.
- Protein expression was detected with Western Blot.
- Immunofluorescence staining was performed on brain sections.
- Gene expression was analyzed using qPCR, NanoString, RNA-Seq and bioinformatics tools such as Galaxy web platform, MetaboAnalyst 5.0, and IPA Qiagen.

❑ *In vitro*

- Myelin phagocytosis assays were performed with bone marrow derived macrophages (BMDMs) and purified myelin extracted from mice.

Results

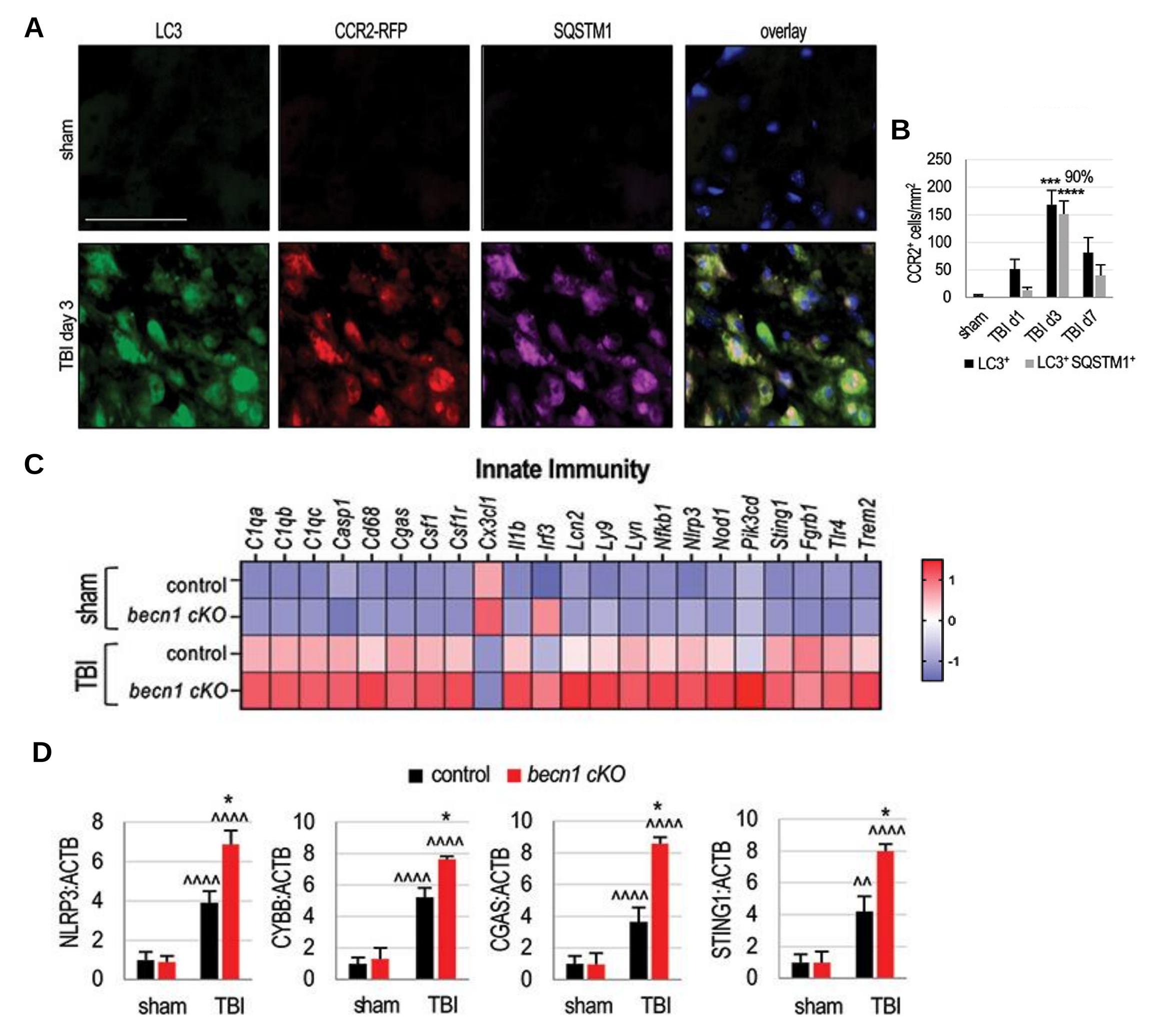


Figure 2. Autophagy inhibition in activated macrophages/microglia contributes to neuroinflammation after TBI. A) 20X images (scale bar: 50 µm) of sham and TBI cortical sections from *Ccr2-RFP* mice stained with autophagy markers LC3 (green) and SQSTM1 (purple). **B)** Corresponding quantification of macrophages (CCR2+) expressing LC3 (black bars) and LC3 with SQSTM1 (gray bars). **C)** Nanostring-based heatmap shows increased expression innate immunity genes in the injured cortex of *becn1 cKO* compared to control mice at 3 days post injury. Color coding was based on z-score scaling. **D)** Densitometric analysis shows increased protein levels of NLRP3, CYBB, CGAS and STING1 in the injured cortical tissue of *becn1 cKO* mice compared to control mice at 3 days post TBI. Data are mean ± SEM; n = 4–5 mice/group. ***p < 0.001, ****p < 0.0001 vs. corresponding sham (one-way ANOVA with Dunnet's post-hoc for multiple comparisons) for (**B**). *p < 0.05, ***p < 0.001, vs. control (genotype effect); ^p < 0.05, ^^p < 0.01, ^^^^p < 0.0001 *vs.* corresponding sham (injury effect); two-way ANOVA with Tukey's post-hoc for multiple comparisons for **(D).**

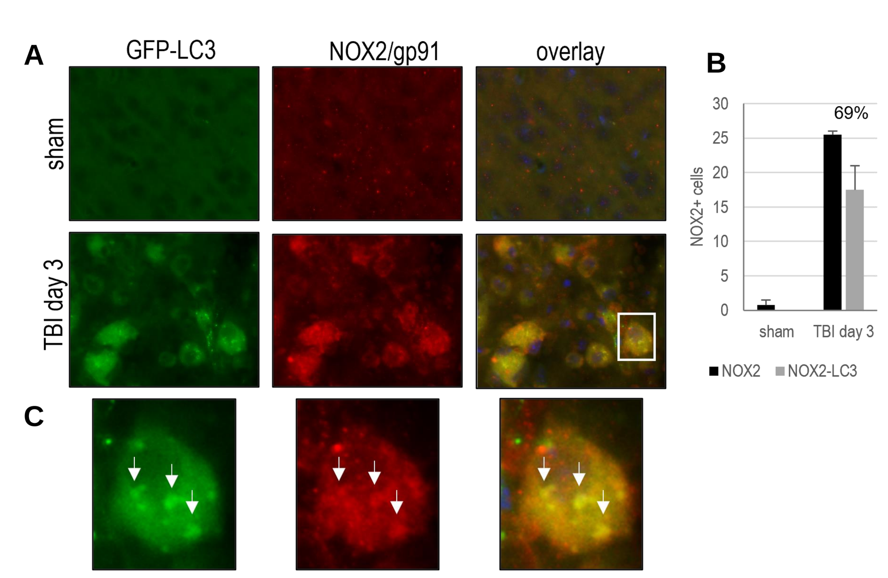


Figure 3. TBI leads to induction of LC3-associated phagosomes (LAPosomes) formation in macrophages/microglia. A) 20x images of cortical brain sections from sham and TBI day 3 *GFP-LC3* mice stained with NOX2/gp91(red). **B)** Quantification of NOX2-LC3 co-localization from **(A)**. **C)** Higher magnification of images in **C** demonstrating co-localization of NOX2 to GFP-LC3 positive vesicles, marking them as LAPosomes. Data are presented as mean ± SEM; n = 4-5 mice per group, * p < 0.05, vs. control (genotype effect); ^p < 0.05, ^^p < 0.01 vs. corresponding sham (injury effect) using two-way ANOVA with Tukey's post-hoc for multiple comparisons.

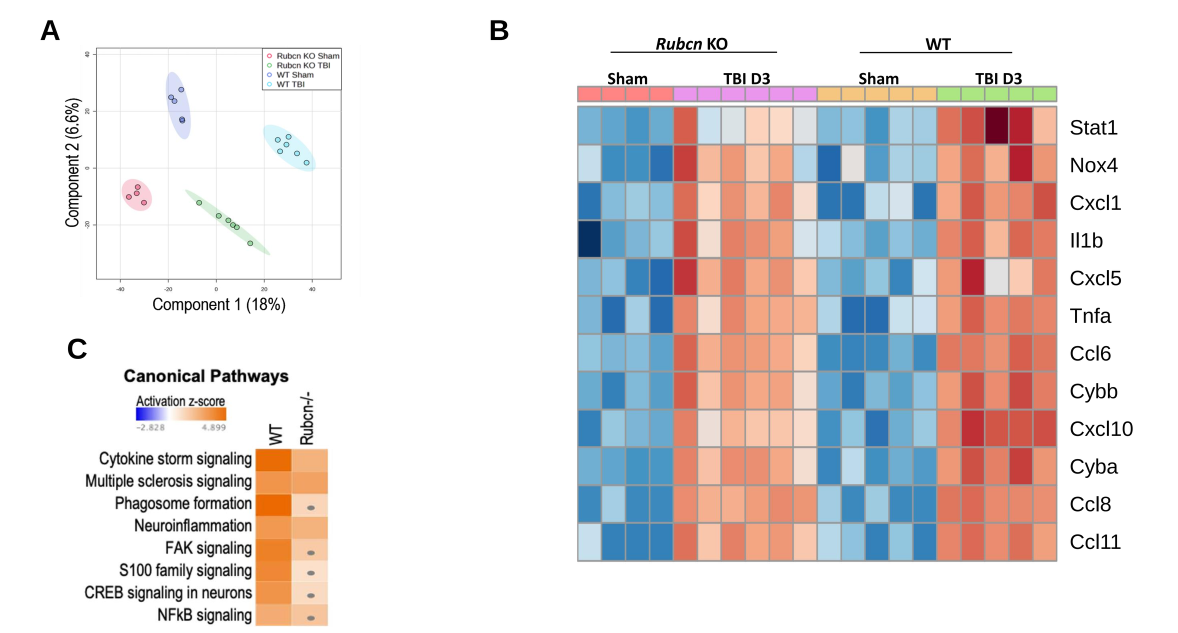


Figure 4. LAP-deficient (*Rubcn* KO) mice displayed less inflammation following TBI. A) Partial Least Square – Discriminate Analysis (PLS-DA) of gene expression in the ipsilateral cortex of sham versus TBI D3 in WT and *Rubcn* KO mice. The 95% confidence interval for each condition is highlighted by the shaded region. Data were normalized and log transformed. **B)** Heatmap of inflammatory genes between *Rubcn* KO and WT groups. Data represent $\log_{10}$ fold change. **C)** Upregulated canonical pathways by the TBI groups analyzed using Qiagen Ingenuity Pathways Analysis (IPA). (n = 4-6 mice per condition)

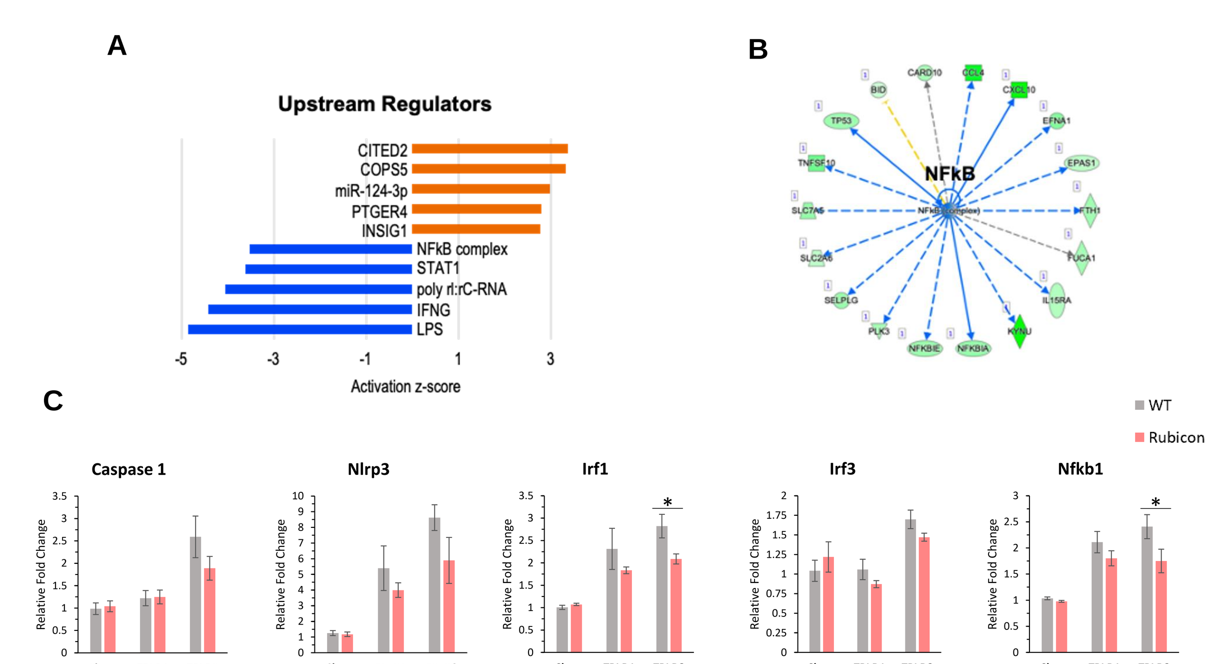


Figure 5. TBI-induced neuroinflammatory responses are attenuated in LAP-deficient mice. A) Predicted upstream regulators identified in Rubicon KO TBI mice in comparison to WT TBI mice. **B)** Network analysis showing that lower expression of genes (highlighted in green) in *Rubcn* KO TBI samples were attributed to predicted downregulation of the NFκB complex. **(A)** and **(B)** were generated by IPA. **C)** Changes in inflammatory gene expression in the ipsilateral cortex of sham vs. TBI D1 vs. TBI D3 in WT and Rubicon KO mice observed by qPCR. Data are mean ± SEM; n = 3–6 mice/group. *p ≤ 0.05

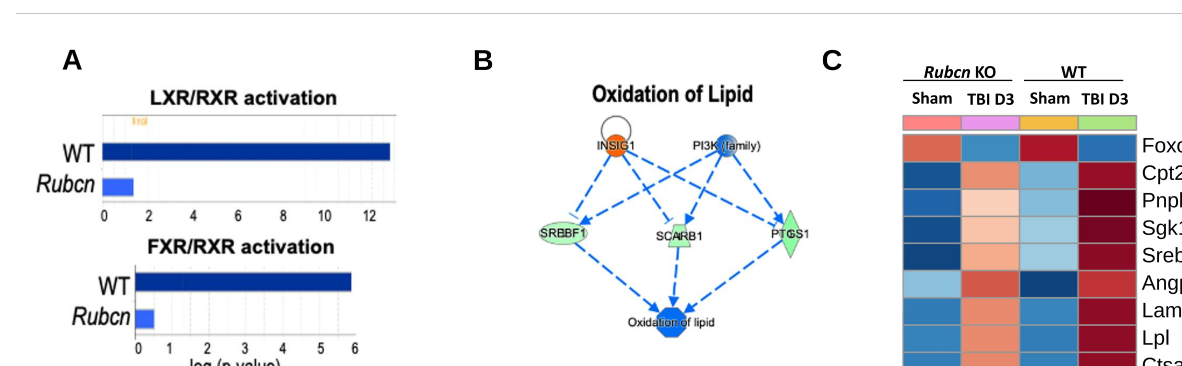


Figure 6. Lipid metabolism pathway is downregulated in LAP-deficient mice after TBI. A) Activation of lipid-related pathways due to TBI effect on WT is more pronounced than that on *Rubcn* KO mice. **B)** "Oxidation of lipid" is inferred to be inhibited in *Rubcn KO* TBI D3 due to decrease in expression of genes (highlighted in green). **C)** Heatmap of-

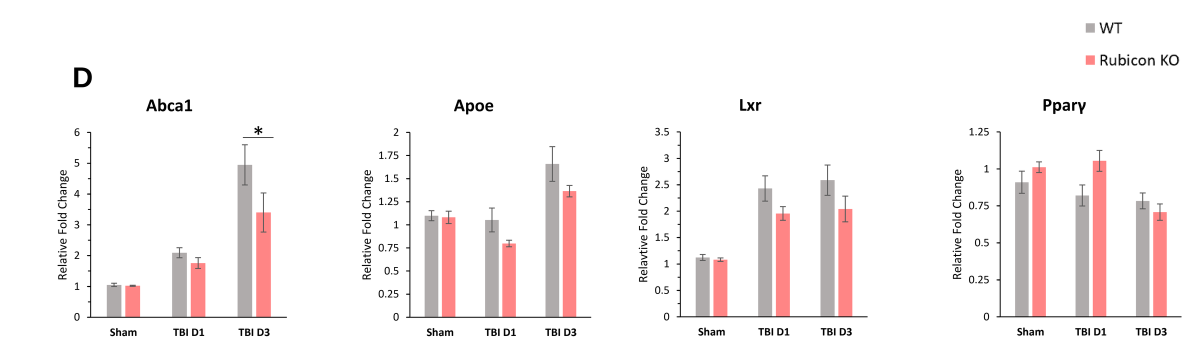


Figure 6 (*continued*). C) lipid-associated genes altered upon TBI in WT and Rubicon KO mice. Data represent $\log_{10}$ fold change. **D)** Expression of lipid-related genes in the ipsilateral cortex of sham vs. TBI D1 vs. TBI D3 in WT and Rubicon KO mice observed by qPCR. Data are mean ± SEM; n = 3–6 mice/group. *p ≤ 0.05

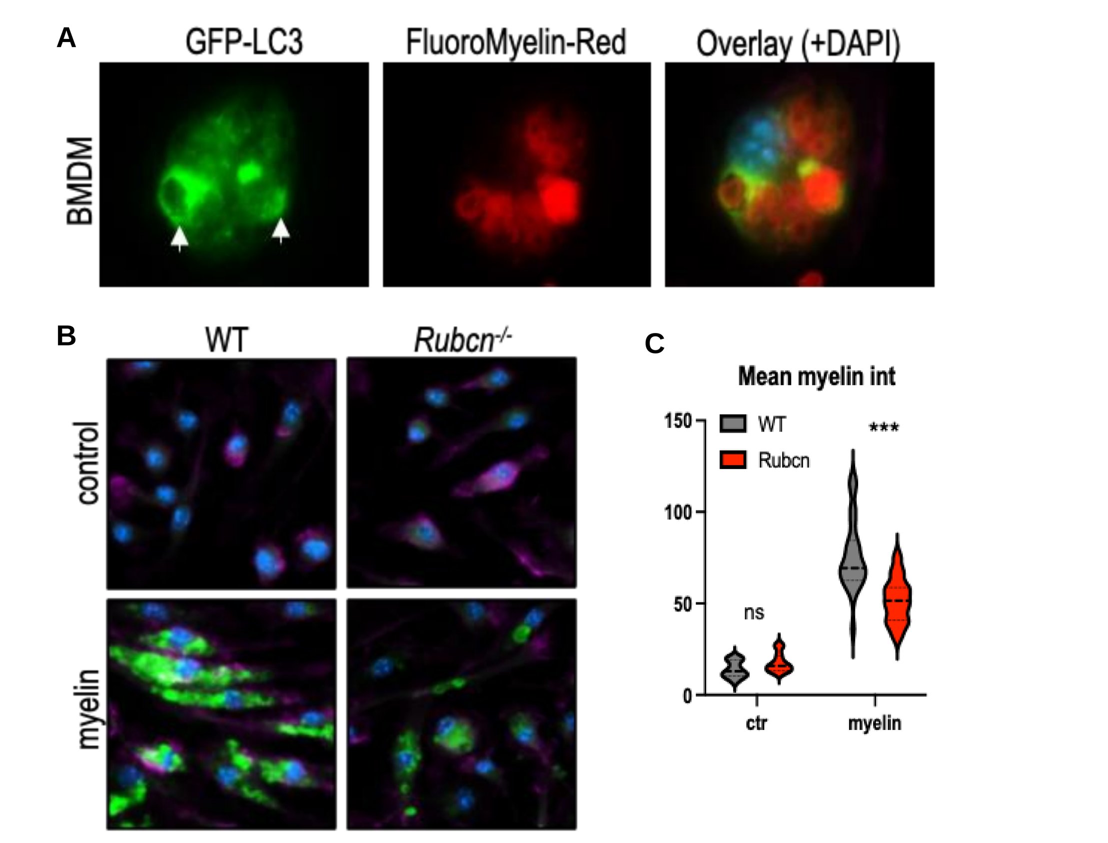


Figure 7. LAP-deficient macrophages phagocytose less myelin debris. A) LC3 (green) in GFP LC3$^{(tg/tg)}$ BMDMs is recruited to the vesicles containing phagocytosed myelin (red). BMDMs were treated with 100 µg of myelin, then stained for FluoroMyelin Red. **B)** WT and *Rubcn* KO BMDMs were treated with 100 µg of myelin for 4 hrs., then stained for FluoroMyelin Green. **C)** Intensity of FluoroMyelin in **(B).** Data represent mean ± SEM (n = 4-5); ***p-value ≤ 0.001.

Conclusion

- TBI induces the formation of LAPosomes in cortical macrophages/microglia.
- LAP-deficiency contributes to attenuated neuroinflammation following TBI.
- Activation of lipid metabolism after TBI is affected by LAP-deficiency.
- Future Direction:
  - Observe changes in protein levels of LAP-deficient mice after TBI.
  - Analyze autophagy flux after TBI from cortical sections.
  - Determine the effect of Rubicon on TBI functional outcomes.

Acknowledgements

The research study was funded by NIH grants R33 AG076858 and RO1 NS115876.