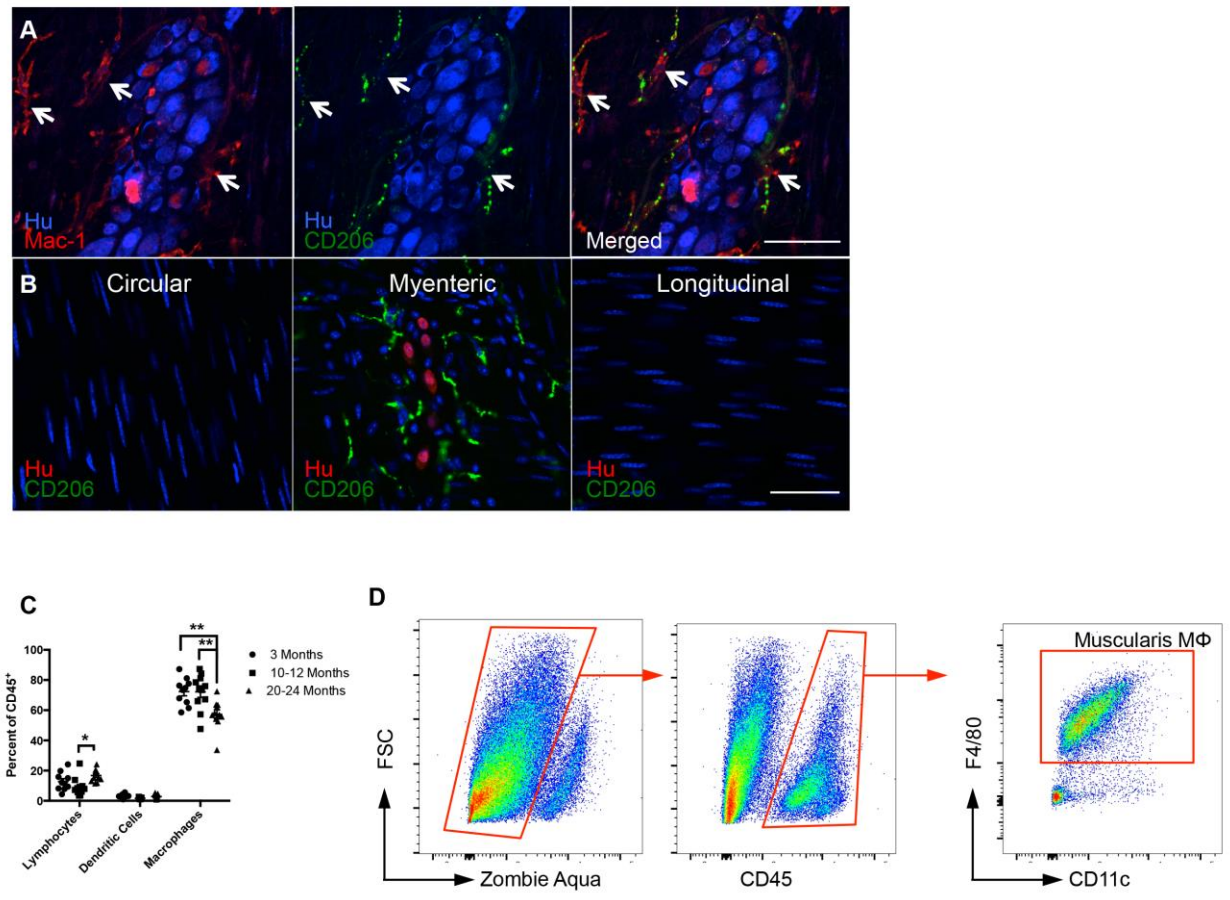
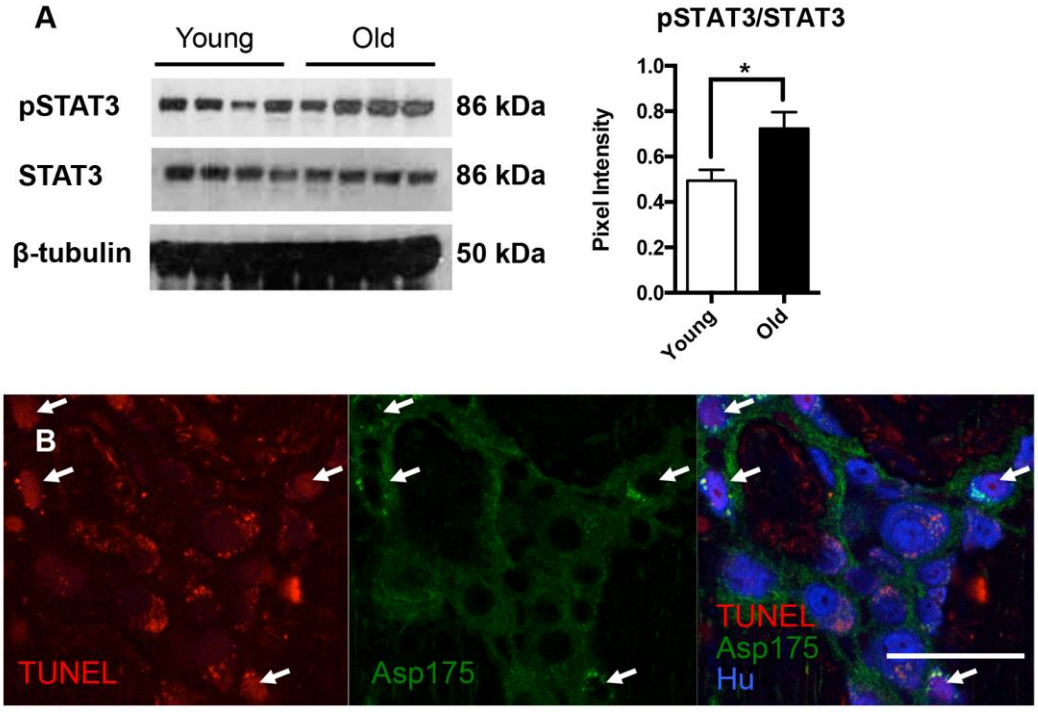
Supplemental Figures

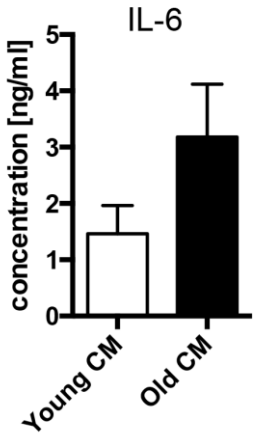
Supplemental Figure 1. Immunostaining and flow cytometry of macrophages and immune cells. Immunostaining of LMMP with the macrophage markers Mac-1 (CD11b, red) and CD206 (green) revealed the majority of MMs (arrows) to co-express both markers (A). While the representative image is from a young mouse, this finding was also observed in old mice. CD206-expressing MMs (green) were observed surrounding Hu^+ neurons (red) in the myenteric plexus layer but not in the circular muscle or longitudinal muscle layers (B). Expressed as percent of $CD45^+$ cells, a statistically significant rise in lymphocytes and fall in macrophages was found in LMMP from old mice compared to young (C). There was no difference in dendritic cells between age groups. For sorting of MMs, sequential gating was performed on single cell suspensions of LMMP for live cells (based on exclusion of Zombie Aqua uptake) then immunoreactivity to CD45 before sorting $F4/80^+$ cells (D). *p<.05, **p<.01 by one-way ANOVA with Bonferroni's multiple comparisons test. Scale bars, 50 μm.



Supplemental Figure 2. pSTAT3 western blot analysis and TUNEL assay on LMMP. Elevated expression of pSTAT3 relative to STAT3 was observed in LMMP lysate from old mice compared with young by western blot (A). Based on pixel intensity, this difference in pSTAT3/STAT3 ratio was statistically significant. TUNEL assay was performed on whole mount LMMP followed by immunostaining for cleaved caspase-3 (Asp175, green) and Hu (blue) (B). $TUNEL^{+}$ neurons (red) were observed to be immunoreactive for Asp 175 (arrows). *p<.05 by t-test. Scale bars, 50 μm.

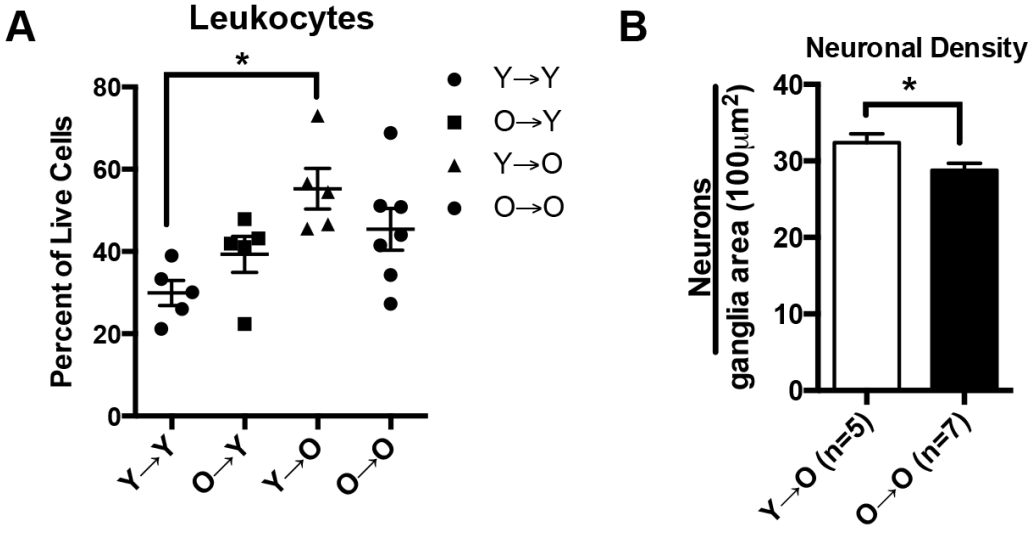


Supplemental Figure 3. IL-6 levels in conditioned media.

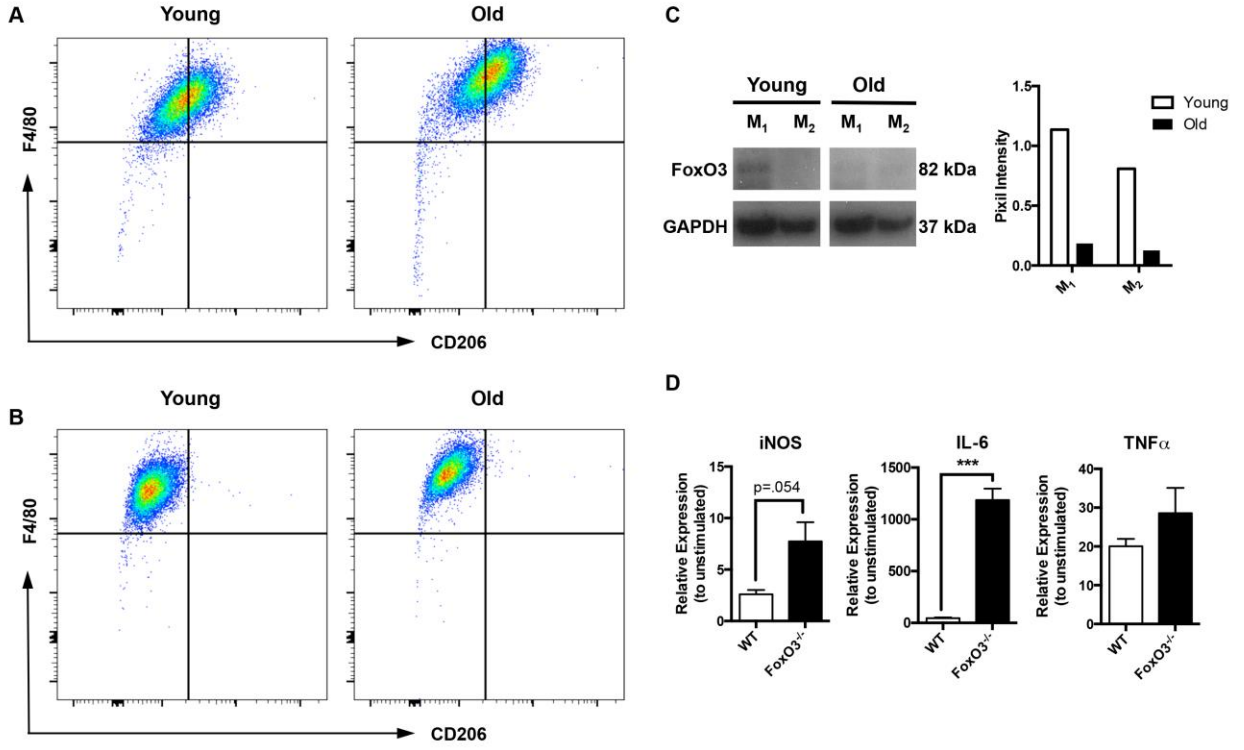


Luminex immunoassays performed on conditioned media revealed levels of IL-6 to be nearly 2-fold greater in Old CM compared to Young CM.

Supplemental Figure 4. Leukocyte infiltration and neuronal density in BM chimeras. A trend towards increased $CD45^+$ leukocytes (as a percent of live cells) was found in the ENS of young mice transplanted with old BM (O→Y) compared to young BM (Y→Y) (A). There was a modest but statistically significant increase in neuronal density found in the myenteric ganglia of Y→O compared to O→O (B). *p<.05 by one-way ANOVA with Bonferroni's multiple comparisons test and t-test.



Supplemental Figure 5. Flow cytometry and expression analysis of BMDMs



Flow cytometry performed on BMDMs from young or old mice treated with either IL-4 and IL-13 (A) or IFNγ (B) revealed similar scatter plots based on F4/80 and CD206 expression. An age dependent reduction of FoxO3 expression was detected in IFNγ-treated (M_1) and IL-4/IL-13-treated (M_2) BMDMs by Western blot with pixel intensity normalized to GAPDH represented as bar chart on right (C). IFNγ-treated (M_1) BMDMs from *FoxO3*$^{-/-}$ mice stimulated with LPS (100 ng/ml) for 4 hours demonstrate increased expression of pro-inflammatory markers relative to their unstimulated state, particularly for IL-6, when compared to WT (D). ***p<.001 by t-test.

Supplemental Figure 6. Model for age-associated motility disorders. An age-dependent decline of FoxO3 in MMs results in loss of suppressive phenotype. MMs assume a pro-inflammatory state resulting in inflammation-induced loss of enteric neurons and ENSCs, and disruption of neuromuscular function.