MYELOPEROXIDASE LOSS OF FUNCTION LEADS TO INTESTINAL DYSBIOSIS AND INFLAMMATION

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Abstract

Background: Myeloperoxidase (MPO), predominantly expressed in neutrophils, catalyzes the production of hypochlorous acid (HOCl) for microbial killing. Due to the redundancy of the immune system in critical functions, the lack of MPO function only results in mild infections, often by fungal pathogens. The significance of MPO in host defense and overall human health remains incompletely understood. In this report, we have investigated how MPO gene-knockout ($MPO^{-/-}$) in mice affects their intestinal microbiota, inflammation state, and transit function.

Methods: $MPO^{-/-}$ and wild-type (WT) mice, co-housed in the same environment for 4 weeks, were sacrificed to harvest their intestines (large and small). Then, the tissues were homogenized for microbial DNA isolation using the QIAamp DNA Microbiome kit. The purified and enriched bacterial DNAs were subjected to PCR amplification and sequencing of the 16S ribosomal DNA hypervariable V3 and V4 regions. Microbiome compositions and diversities were analyzed and compared. Furthermore, $MPO^{-/-}$ and WT mice were investigated for their mucosal immune cell compositions in the small intestines via flow cytometry, level of fecal inflammatory marker (calprotectin) via ELISA, and bowel movement via carmine red passing assay.

Results: A total of 13 families of bacteria were found in the intestinal microbiota, of which 2 families (Peptostreptococcaceae and Erysipelatoclostridiaceae) were unique to $MPO^{-/-}$ mice, while 7 families were unique to WT mice, including Ruminococcaceae, Desulfovibrionaceae, and Bifidobacteriaceae. Alpha diversity metrics (Shannon entropy, Observed features, Faith PD) were significantly higher in the WT samples than in the $MPO^{-/-}$ ones. Beta diversity metrics demonstrated a significant difference between the genotypes. Moreover, the small intestines of $MPO^{-/-}$ mice were highly inflamed, reflected by significantly more infiltrations of neutrophils and T cells in the mucosa. Fecal calprotectin was significantly higher in $MPO^{-/-}$ mice. Physiologically, $MPO^{-/-}$ mice had a significantly slower bowel movement rate.

Conclusions: MPO loss of function induces dysbiosis, inflammation, and slow movement of the intestines. Thus, MPO functions as a protective factor against infection and inflammation in normal intestinal health.

Materials and Methods

Mice and Intestinal Microbial DNA Isolation: Intestinal samples from $MPO^{-/-}$ and WT mice (6-8 weeks old; 4 animals per genotype) were dissected, homogenized, and the microbial DNA was isolated. The protocol was designed to enzymatically deplete host DNA prior to bacterial cell lysis. An optimized combination of mechanical and chemical lysis was used to reduce bias introduced by the differential susceptibility of bacterial cell walls to the lysis procedure.

Microbial 16s rRNA Gene Sequencing and Diversity Analysis: The purified and enriched bacterial DNAs were submitted for 16S rRNA gene sequencing. To effectively align V3 and V4 sequencing fragments and trim low-quality reads, the dada2 denoise-paired method was employed. Following quality control, a substantial number of paired reads were retained for each sample for subsequent analysis. Diversity analysis was carried out utilizing the q2-diversity plugin. Taxonomic analysis was performed using q2-feature-classifier plugin and Silva-138-99-nb-classifier. Differential abundance (DA) of taxonomic composition at different levels across the samples were calculated using ANalysis of COmpositions of Microbiomes (ANCOM) method via q2-composition plugin. All generated data were visualized in Qiime2 View.

Feces collection and measurements of MPO and calprotectin: Experimental mice were individually placed in sterile single containers, and their fecal pellets were collected. MPO and calprotectin levels in fecal pellets were measured with mouse myeloperoxidase and Calprotectin DuoSet ELISA kits according to manufacturer recommended protocols.

Immune cell composition in intestinal mucosa: Intestines of WT and $MPO^{-/-}$ mice were isolated, longitudinally cut open, and cleansed by flushing out the contents. Then, the tissues were minced and placed in culture medium for digesting. The digested tissues were filtered and resuspended for immunostaining. The cells were immunostained on ice in the dark for 40 minutes using a panel of antibodies or fluorescent dyes: CD45, Fixable Viability Stain 780, Brilliant Stain Buffer Plus, CD11B, F4/80, CD19, CD3ε, Ly6C, and Ly6G. After post-staining fixation, the cells were subject to flow cytometry.

Intestinal transit time measurement: Oral gavage solution was made by dissolving 6% (w/v) Carmine Red dye and 0.5% (w/v) methylcellulose in hot autoclaved mouse drinking water. After weighing each mouse, the solution was administered according to body weight (10 ml/kg). Then, fecal pellets were collected every 10 minutes. GI transit time was calculated from the moment of gavage completion (T0) to the time of the first appearance of a fully-red fecal pellet.

Statistical Analysis: For most experiments, the 2 group comparisons were conducted using a two-tailed, unpaired Student's t-test. The data were presented as mean± standard deviation (SD). A significance level of $P < 0.05$ was considered statistically different. Statistical analyses and graphical representations were performed using GraphPad Prism 10, Microsoft Excel 2016, or R programming (version 4.3.1).

Results

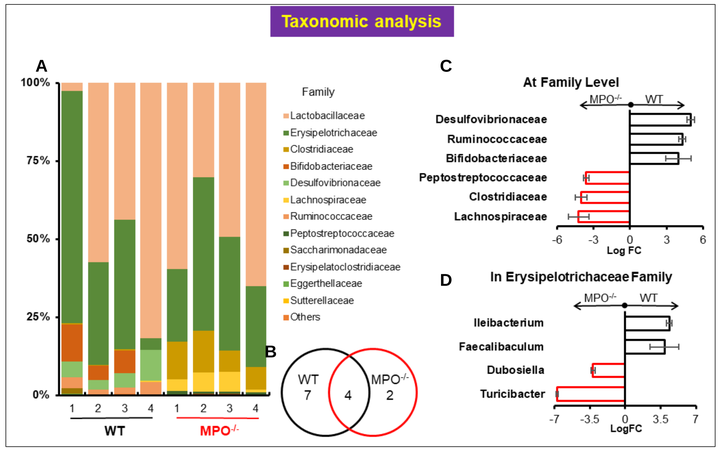

Figure 1. Taxonomic composition and bacteria differential abundance shows differences between the WT and $MPO^{-/-}$ gut. (A) The bacterial compositions in the intestinal content of WT and $MPO^{-/-}$ mice on family level. (B) Venn diagram showing the number of bacterial family in the intestinal microbiota of WT and $MPO^{-/-}$ mice. (C & D) Differential abundance (DA) analysis of bacteria between WT and $MPO^{-/-}$ groups. (C) DA analysis at family level. (D) DA analysis at genus level in the family of *Erysipelotrichaceae*. The analyses were performed by Qiime2 plugin with ANCOM method ($p < 0.05$, $q < 0.05$). FC, fold-change.

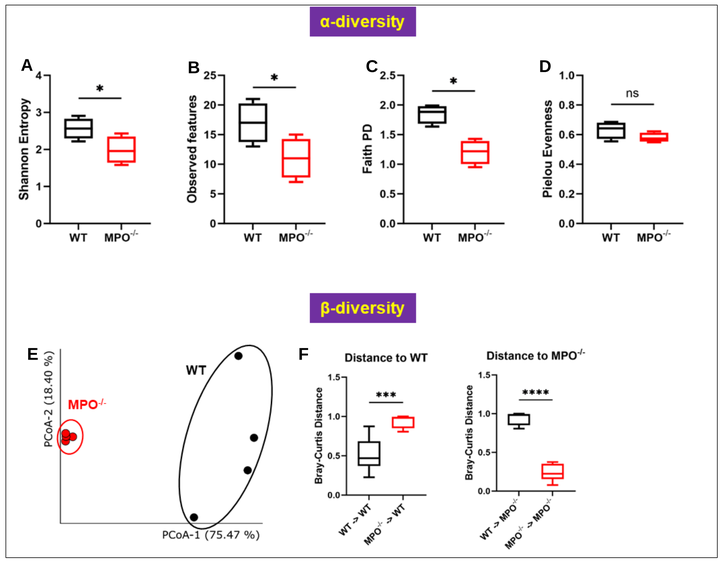

Figure 2. Bacterial community diversity differs between the WT and $MPO^{-/-}$ gut. (A-D) Alpha diversity measured by different methods: Shannon Entropy (A), Observed features (B), Faith PD (C), and Pielou Evenness (D). (E-F) Beta diversity measured by Bray-Curtis method. (E) Principal coordinate analysis (PCoA) of the microbial community based on Bray-Curtis distance matrix. (F) The Bray-Curtis distance among samples intra- and inter-groups. Significant differences were determined by a 2-tailed, unpaired Student's t-test. *p<0.05, ***p<0.001, ****p<0.0001.

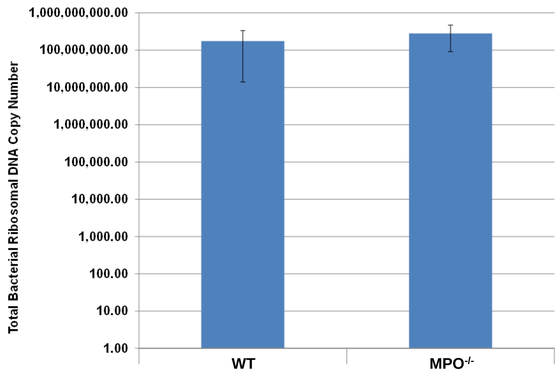

Figure 3. Quantification of total intestinal bacterial loads shows no significant difference. Quantitative PCR was performed to compare the total bacterial ribosomal DNA copy numbers in WT and $MPO^{-/-}$ mouse intestines, which indicates the total bacterial numbers in the guts.

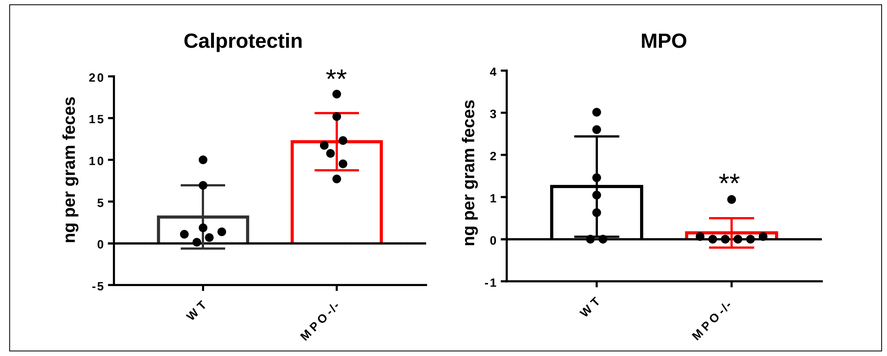

Figure 4. The intestines of $MPO^{-/-}$ mice have a significantly higher degree of inflammation compared to WT. Fecal calprotectin and MPO levels (ng/g feces) were measured via ELISA. Statistical differences were determined by unpaired Student's t-test (n=7 per group). Results are expressed as means ± SD. **P < 0.01.

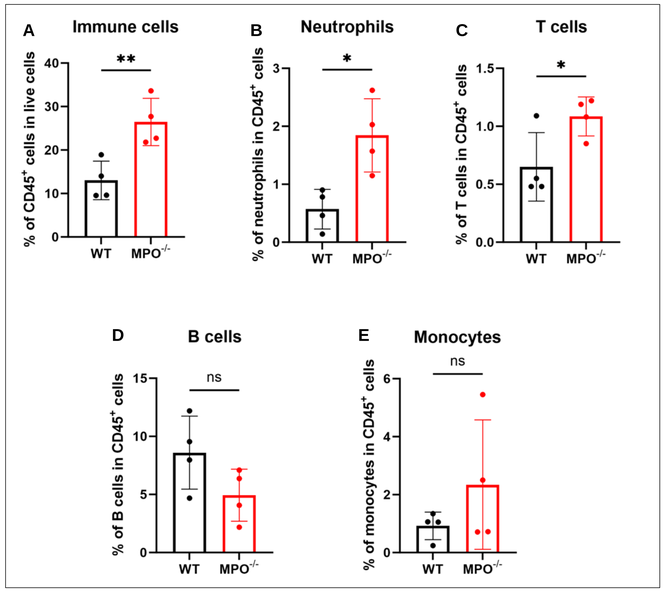

Figure 5. Differential of immune cells isolated from intestinal lamina propria show significant differences between WT and $MPO^{-/-}$ mice. (A) Percentage of immune cells ($CD45^+$) in all live lamina propria cells. (B) Percentage of neutrophils ($CD11b^+Ly6C^+Ly6G^+$) in $CD45^+$ cells. (C) Percentage of T cells ($CD11b^-CD3\varepsilon^+$) in $CD45^+$ cells. (D) Percentage of B cells ($CD11b^-CD19^+$) in $CD45^+$ cells. (E) Percentage of monocytes ($CD11b^+Ly6C^+Ly6G^-$) in $CD45^+$ cells. Data were presented as mean±SD. A 2-tailed, unpaired Student's t-test were used to determine statistic differences. *p<0.05, **p<0.01, ns: not significant.

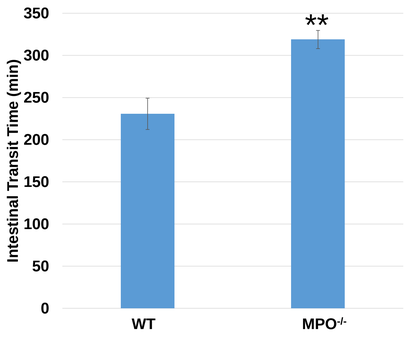

Figure 6. Carmine red dye passing shows significant difference in fecal transit time. WT and $MPO^{-/-}$ mice were measured for gut transit from the time of Carmine red gavage (T0) to the time of the first completely-red fecal pellet. Statistical differences were determined by unpaired Student's t-test (n=7, p<0.01).

Conclusions

- $MPO^{-/-}$ mice develop intestinal dysbiosis, despite no significant difference in total bacterial number as compared to WT mice.
- $MPO^{-/-}$ mice are inflamed in intestines, which is reflected by a higher fecal calprotectin level and higher percentage of immune cells, including neutrophils and T-cells.
- $MPO^{-/-}$ mouse intestines move significantly slower than the WT ones.
- In summary, this research demonstrates that MPO is protective against intestinal infection and inflammation.

Acknowledgements

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