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An intact microbiota is required for the gastrointestinal toxicity of the immunosuppressant mycophenolate mofetil

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Short Title: Role of the microbiota in MMF toxicity

KEYWORDS: immunosuppressive drugs; microbiome; transplantation; intestine; germ-free; mycophenolate mofetil; colitis; gut; immunosuppression

BACKGROUND: Mycophenolate mofetil (MMF) is commonly prescribed after transplantation and has important advantages over other immunosuppressive drugs but frequent gastrointestinal (GI) side effects limit its use. The mechanism(s) underlying MMF-related GI toxicity have yet to be elucidated.

METHODS: To investigate MMF-related GI toxicity, mice were fed chow containing MMF (0.563%) and multiple indices of toxicity, including weight loss and colonic inflammation, were measured. Changes in intestinal microbial composition were detected using 16S rRNA Illumina sequencing and downstream PICRUSt analysis was used to predict involved metagenomic pathways. Germ-free (GF) mice and mice treated with orally-administered broad-spectrum antibiotics (ABX) were utilized to interrogate the importance of the microbiota in MMF-induced GI toxicity.

RESULTS: Mice treated with MMF exhibited significant weight loss, related to loss of body fat and muscle, and marked colonic inflammation. MMF exposure was associated with changes in gut microbial composition, demonstrated by a loss of overall diversity, expansion of *Proteobacteria* (specifically *Escherichia/Shigella*) and enrichment of genes involved in lipopolysaccharide (LPS) biosynthesis which paralleled increased levels of LPS in the feces and serum. MMF-related GI toxicity was dependent on the intestinal microbiota, as MMF did not induce weight loss or colonic inflammation in GF mice. Furthermore, ABX prevented and reversed MMF-induced weight loss and colonic inflammation.

CONCLUSIONS: An intact intestinal microbiota is required to initiate and sustain the GI toxicity of MMF. MMF treatment causes dynamic changes in the composition of the intestinal microbiota that may be a targetable driver of the GI side-effects of MMF.

Introduction

Following transplantation, potent immunosuppressive drugs (ISDs) are required to prevent recipient immune activation and allograft injury. Mycophenolate mofetil (MMF) is a prodrug whose active metabolite mycophenolic acid (MPA) inhibits inosine monophosphate dehydrogenase and suppresses the proliferation of T and B lymphocytes (1). MMF is the anti-proliferative agent of choice in combination with a calcineurin inhibitor for maintenance immunosuppression post-heart and lung transplantation (2). However, despite the proven advantages of MMF therapy, frequent gastrointestinal (GI) side effects limit its use and tolerability. MMF toxicity affects 45% of patients and ranges in severity from nausea, vomiting, diarrhea and abdominal pain to weight loss and colitis resembling inflammatory bowel disease (3, 4) (5) (6). MMF-related GI toxicity can lead to dosage reduction or discontinuation, which has been associated with rejection and allograft loss (7) (8) (9).

While the mechanism(s) contributing to MMF-related GI toxicity have yet to be well characterized, we recently reported a clinical case wherein shifts in the intestinal microbiota were associated with the onset of GI side-effects (10). The gut microbiota, referring to all of the microorganisms in this niche, and its associated metagenome (microbiome) influences diverse aspects of host physiology and changes in its composition impacts host health (11, 12). The stability of the microbiota is associated with positive health outcomes, while disruption has been linked to disease (13, 14). Medications, including ISDs, can alter the gut microbiota (15, 16) and even trigger intestinal dysfunction and inflammation (17-19). Changes in the microbiota following solid organ transplantation (SOT) have been reported (10, 20-22), but the influence of ISDs on the microbiome remains understudied. In the present study, we interrogated the effect of MMF exposure on the gut microbiome and investigated the role the microbiota plays in MMF-induced GI toxicity in mice.

Methods

Animal studies

Specific pathogen-free (SPF) male C57BL/6, B6.129S7-Rag1<*tm1Mom*>/J (*Rag1-/-*) and BALB/c mice between 7 and 10 weeks of age (Jackson Laboratories) were housed in SPF conditions in sterilized, filter-top cages. Littermates were used throughout all studies to control for differences in the composition of the intestinal microbiota. Germ-free (GF) C57BL/6 mice were generated by two-stage embryo transfer, as previously described (23) and bred and maintained in flexible film isolators in McMaster University's Axenic/Gnotobiotic Unit. GF status was monitored during and at the conclusion of the experiment by anaerobic and aerobic culture, SYTOX green stain and PCR. All procedures were approved by the Health Sciences Animal Care Committee at the University of Calgary or the Animal Research Ethics Board at McMaster University and conform to guidelines established by the Canadian Council on Animal Care.

Standard chow supplemented with 0.563% MMF, a dose previously shown to suppress the proliferation of murine lymphocytes without significant liver or kidney toxicity (24), was formulated by Envigo and fed to mice *ad libitum*. Standard chow without MMF (Envigo) was used as the control diet. At the beginning of each experiment, mice were switched from standard chow provided by the Central Animal Facility at the University of Calgary to either diet from Envigo. For GF studies, diets were autoclaved at 132°C for 40 mins (20 mins steam, 20 mins dry) to ensure sterility. To deplete the intestinal microbiome, mice were exposed to ampicillin (1 g/L), metronidizole (1 g/L), neomycin (1 g/L) and vancomycin (500 mg/L) combined in the drinking water for two weeks (25). Body composition was measured by quantitative nuclear magnetic resonance using a Minispec LF-110 NMR Analyzer (Bruker Optics, Milton, ON, Canada). MPA and mycophenolic acid glucuronide (MPAG) were measured using ultra-high performance liquid chromotography and mass spectrometry (LCMS; a full explanation of methods can be found in the supplementary materials online at jhltonline.org).

Detection of colonic inflammation

Myeloperoxidase (MPO) activity was measured in colonic tissue homogenates, as previously described (26). Cytokine levels were assessed in colonic homogenates and serum using the Mouse Cytokine/Chemokine 31-Plex Discovery Assay (Eve Technologies). Intestinal permeability was assessed by detection of lipopolysaccharide (LPS) in serum using the E. TOXATE^{TM} kit (Sigma-Aldrich). LPS was also quantified in feces using human embryonic kidney (HEK)-Blue-mTLR4 cells (Invivogen), as previously described (27) . Procedures for isolation of lamina propria cells, flow cytometry and antibody clones used are found in the supplementary materials online at jhltonline.org.

Bacterial DNA isolation, Illumina sequencing and bioinformatics

Procedures for bacterial DNA isolation, Illumina sequencing, operational taxonomic unit (OTU) table construction and downstream analysis, including the use of PICRUSt 1.0.0, are found in the supplementary methods online at jhltonline.org.

Statistics

Statistical analyses were performed on data expressed as mean ± SEM, where *n* is the biological replicate (number of mice per group). Data were analyzed using either Student's *t*-test, one-way ANOVA with appropriate post hoc analysis, or two-way ANOVA. Analyses were performed using GraphPad Prism 7; in all cases, $P < 0.05$ was considered significant.

Results

MMF exposure causes rapid weight loss and macroscopic changes in the colon

To characterize the GI-related effects of MMF treatment, we examined multiple parameters after administering MMF to mice. Oral administration of MMF over the course of 8 days led to rapid and significant weight loss (loss of ~25% of body weight by day 8; 76.2 ± 0.5% of initial weight) when compared to control mice (Fig. 1A). After withdrawing MMF, mice began to regain lost weight, but never fully recovered to the weight of control mice. These changes were not the result of decreased food or water intake, as chow (Fig. S1A) and water consumption were not significantly different between groups. Significant decreases in fat mass and lean muscle mass, but not free fluid mass, were also observed in MMF-exposed mice (Fig. S1B). Accompanying the loss of body fat and lean muscle was significant colon shortening in MMF-exposed mice after 8 days (Fig. 1B,C) that recovered to control length by day 16 following MMF withdrawal for 8 days (Fig. 1C). MMF treatment also led to a substantial decrease in cecum weight, spleen weight and hematocrit (Fig. 1B, D-F). Interestingly, after withdrawal of MMF, cecum weight and hematocrit both recovered but animals developed splenomegaly, with spleens >2-fold larger than those of control mice (Fig. 1D-F).

MMF exposure causes colonic inflammation

In comparison to control animals, MMF-treated mice had significantly higher levels of intestinal granulocyte infiltration as measured by MPO activity (Fig. 2A) that normalized after the withdrawal of MMF. Overall, when compared to control mice, colonic tissue from mice exposed to MMF for 8 days demonstrated elevated levels of several proinflammatory mediators including interleukin (IL)-6, IL-1β, TNF-α, and the chemokines KC (CXCL1), macrophage inflammatory protein 2- alpha (MIP-2α/CXCL2), macrophage inflammatory protein 1β (MIP-1β), and monocyte chemoattractant protein 1 (MCP-1/CCL2). (Fig. 2B). MMF-induced inflammation was

characterized by infiltration of neutrophils (defined as $CD45^{+}MHCII^{-}CD11b^{+}Ly6C^{int}Ly6G^{+}$) (28, 29) and inflammatory monocytes (defined as $CD45^{+}MHCII^{-}CD11b^{+}Ly6C^{Hi}$ cells) (30) (Fig. 2C&D), but no change in the number of eosinophils (Fig. S2B&C). Evidence of increased inflammation occurred in the absence of gross microscopic mucosal damage (Fig. S3).

Intestinal toxicity of MMF occurs independently of adaptive immune cells and mouse strain

MMF is thought to selectively target B and T cells to suppress allograft-targeted immune responses (1). To determine whether the MMF-related GI toxicity was due to an effect on these cell populations, we performed experiments in *Rag1*$^{-/-}$ mice (which lack mature B and T cells). We found that *Rag1*$^{-/-}$ mice displayed similar weight loss to wild type animals when treated with MMF (Fig. 3A). MMF-treated *Rag1-/-* mice also exhibited colon shortening (Fig. 3B), decreased cecum weight (Fig. 3C), decreased spleen weight (Fig. 3D) and reduced hematocrit (Fig. 3F). The physiological and inflammatory changes induced by MMF were not strain-specific as BALB/c mice displayed similar changes as C57BL/6 mice when exposed to MMF for 8 days (Fig. S4).

MMF exposure causes changes in the diversity and composition of the fecal microbiota

Given that MMF toxicity occurred independently of lymphocytes and mouse strain, we hypothesized that the toxicity of MMF may be influenced by the intestinal microbiota. We first examined microbiota composition profiles in DNA extracted from fecal pellets collected every four days from mice fed control or MMF-containing chow (8 days on MMF then recovery for an additional 8 days following MMF withdrawal). As both control and MMF-exposed mice were switched to new diets at the beginning of the experiment, comparisons were performed at each time point, to ensure the control diet alone was not inducing large changes. Observed diversity (an α-diversity measure that counts the number of unique OTUs in each sample to determine

richness) measurements revealed that MMF treatment led to a rapid decrease in microbiota richness, as early as 4 days after drug exposure (Fig. 4A). There was also a significant reduction in observed diversity on day 8 during MMF treatment, an effect that continued even after withdrawal of MMF. Control samples remained unchanged for the entire 16-day experiment (Fig. 4A). The Shannon diversity measure of evenness, an estimate of both the richness and the relative abundance or distribution of all OTUs within a sample, did not uncover remarkable differences in the microbiota between control and MMF-treated mice, although there was decreased Shannon diversity in the experimental group at day 16 (Fig. 4A).

Changes in the microbiota were further characterized by examining the distribution of bacterial phyla in each fecal sample. Figure 4B shows stacked histograms depicting the bacterial composition of fecal samples obtained from individual mice every 4 days over the course of the 16-day experiment. The two dominant phyla in mice before MMF treatment were Firmicutes (~80%) and Bacteroidetes. Less abundant phyla included Actinobacteria, Deferribacteres, Proteobacteria, Tenericutes and Verrucomicrobia. The microbiota of control mice appeared relatively stable over the 16-day course of the experiment. However, as early as 4 days after exposure to MMF, reductions in the Bacteroidetes and Verrucomicrobia phyla were detectable and the relative abundance of Proteobacteria was increased in a number of MMF-treated mice (Fig. 4B). These changes continued to progress after 8 days of MMF exposure, with a substantial expansion of Proteobacteria seen in all mice along with a detectable expansion in Deferribacteres. When MMF was withdrawn, shifts in the relative abundance of certain phyla continued. On day 12, there was further expansion of Firmicutes (~95%) as Bacteroidetes continued to diminish (<5%). By day 16, Proteobacteria were still present and there was a clear expansion of Actinobacteria (Fig. 4B).

The Bray-Curtis dissimilarity metric was used to compare the microbial diversity between groups over the time course of the experiment. Before administration of MMF-containing chow, all mice showed similar microbial profiles with overlapping clustering (Fig. 4C). After 4 days of

MMF administration, the treated group had shifted away from the control group and, by day 8, the two groups were distinctly separate (Fig. 4C). After withdrawal of MMF, the separation of the groups began to reverse with the MMF-treated group shifting back towards the control group, with no distinct clustering noticeable by day 16. We then examined the abundance of specific bacterial families over the course of MMF administration and its withdrawal. At the family level, we found that changes induced by MMF were widespread with several different patterns of change including both the depletion and enrichment of important intestinal bacterial populations (Fig. S5).

MMF alters the microbiome, increasing the pathogenic potential of the intestinal microbiota

Examination within the Proteobacteria phylum on day 8 following MMF exposure revealed that this phylum was dominated by the genus *Escherichia*/*Shigella* (Fig. 5A). Tracking of *Escherichia/Shigella* (the two of which cannot be resolved using 16S sequencing) over the course of MMF exposure showed that a gradual increase in this genus was associated with weight loss and other symptoms induced by MMF (Fig. 5B). PICRUSt analysis identified an enrichment of genes encoding for enzymes involved in LPS biosynthesis, as identified from the Kyoto Encyclopedia of Genes and Genomes (KEGG) reference pathways (Fig. S6), in the feces of mice after 8 days of MMF exposure (Fig. 5C). These changes were associated with increased bioactive LPS detected in the feces (Fig. 5D) and serum (Fig. 5E) of MMF-treated mice. Along with these changes we also observed a significant loss of the genera *Akkermansia*, *Parabacteroides* and *Clostridium* on day 8 following MMF exposure (Fig. 5F).

MMF GI toxicity requires the presence of a microbiota

To determine whether the MMF-related GI toxicity was dependent on an intact intestinal microbiota, we performed experiments using GF mice. Before initiating the GF experiments, we

confirmed that autoclaved MMF-containing chow maintained its toxicity (Fig. S7). When fed to GF mice, MMF-supplemented chow did not trigger weight loss, nor did it induce colon shortening. Furthermore, MMF-treated GF mice exhibited the same cecum weight and spleen weight as GF mice fed the control diet (Fig.6A-E). Hematocrit and MPO levels were slightly lower in MMF-fed GF mice (Fig. 6F,G) but, in the absence of the gut microbiota, the overt GI toxicity of MMF, including colonic inflammation, like that seen in SPF mice, was not observed.

Antibiotics prevent and reverse the toxicity induced by MMF

To test whether targeting the microbiota could prevent the effects of MMF, intestinal bacteria were depleted using a cocktail of broad-spectrum antibiotics (25) for two weeks prior to the administration of MMF-containing chow. Early antibiotic administration was protective, with ABX-treated mice showing no significant decreases in weight, colon length, spleen weight, or hematocrit (Fig. 7A-C). Pretreatment with ABX also prevented the loss of body fat and lean mass (Fig. 7D). ABX administration did not significantly change serum levels of MPA, MPAG (Fig. 7E) or liver expression of drug transporters involved in the metabolism of MMF (Fig S8). Pretreatment with ABX also prevented colonic inflammation (Fig. 7F) and inflammatory mediator release in the serum and colon (Fig. S9). Taking an interventional approach, we next assessed whether ABX could treat/reverse established MMF-related GI toxicity. Interestingly, after 8 days of MMF exposure, ABX treatment was sufficient to promote significant increases in body weight, colon length, and cecum weight, during continued MMF administration (Fig. S10).

Conclusions

The reported frequency of MMF-related GI side effects ranges between 12-80% (8, 31-34) with 22% requiring dose reduction or discontinuation of the drug (8). With evidence that MMF impacts the gut microbiota (10, 35), we developed a model to study the effects of MMF on the intestinal microbiota and its associated microbiome. In two wild type strains (C57BL/6 and BALB/c), and in *Rag1*-deficient mice, we have shown that MMF treatment leads to rapid and significant weight loss accompanied by gross colonic changes and inflammation, recapitulating the effects seen in humans (36, 37). We also observed that MMF administration prompted alterations in the composition of the microbiota and its associated metagenome. The presence of the microbiota appears to be a critical factor necessary for the toxicity of MMF, as weight loss and colonic pathology were absent in GF mice, and the administration of antibiotics was sufficient to both prevent and reverse the phenotype associated with MMF exposure.

Alterations in the intestinal microbiota have been reported after SOT and hematopoietic stem cell transplantation (HSCT) in both mice and humans, with decreased bacterial diversity being a common finding (21, 38-41). In our mice, we noted a decrease in observed diversity, but we did not see an early change in Shannon Diversity related to MMF treatment. These findings are consistent with a human study showing no change in the Shannon diversity of the intestinal microbiota within the first month after kidney transplantation while patients were maintained on ISDs (42). In addition to changes in diversity, the depletion or enrichment of certain bacteria following transplantation has been reported, with an increase in *Lactobacillales* and a decrease in *Clostridiales* in humans and mice undergoing HSCT (39). An increase in Proteobacteria, accompanied by decreases in Bacteroides, Ruminococcus, Coprococcus and Dorea, was seen in kidney transplant patients with diarrhea, but, given the multiple confounders, it could not be

determined if this was related to ISD administration (21). Recently, we have reported that the use of MMF in a pediatric heart transplant patient appeared to have influenced the gut microbiota with negative consequences for the patient including the development of colonic lymphonodular hyperplasia and hematochezia (10).

The overabundance of certain bacteria can have detrimental effects on the host. We observed that MMF exposure promoted expansion of the phylum Proteobacteria composed mainly of bacteria belonging to the genus *Escherichia/Shigella*. Certain strains of this genus can be pathogenic and are often labelled as pathobionts (43). This increase was accompanied by gene enrichment for multiple (>8) bacterial enzymes involved in the biosynthesis of LPS and increased levels of LPS in mouse feces and serum. A microbiome skewed towards greater LPS production can be indicative of a more invasive microbiota (27) (44), in part because overproduction of LPS can be detrimental to the host by activating innate immunity and triggering proinflammatory gene expression to drive intestinal inflammation (44) (45). The ability of antibiotic treatment to reverse and prevent the GI toxicity of MMF suggests that microbes capable of potentiating significant gut pathology and inflammation may have been eliminated. The absence of MMF toxicity in GF mice also supports the idea that an indigenous microbial driver could either trigger or potentiate MMF toxicity. Loss of beneficial microbes could also play a role in MMF toxicity. For example, increases in Firmicutes and decreases in Bacteroidetes, like those observed in our MMF-exposed mice, have been implicated in intestinal inflammation related to IBD (46). In the current study, we also found a significant decrease in the genera *Akkermansia, Parabacteroides* and *Clostridium* over the course of MMF treatment with almost complete elimination after 8 days of MMF exposure. Each of these bacteria have been shown to promote gut health through a number of distinct mechanisms (47, 48) (49) (50) (51).

In conclusion, our model of MMF exposure recapitulates the human phenotype of MMF-induced GI toxicity. Our findings in mice suggest that MMF induces a dysregulation of the intestinal microbial community with the expansion of potentially harmful bacteria, shifting the

microbiota composition towards one with greater pathogenic potential. Our work also suggests that the GI toxicity caused by MMF requires an intact microbiota and can be prevented or reversed through manipulation of the gut microbiota. Beyond ABX treatment, which can present several clinical complications, other approaches to rationally manipulate the microbiota (eg. probiotics and prebiotics) may prove important in combating the toxicity associated with MMF use. However, the failure of the prebiotic inulin to prevent MMF toxicity (unpublished data) highlights the importance of understanding the specific bacteria, or bacterial components and metabolites involved in MMF toxicity and represents an important area of future clinical research.

Disclosure Statement

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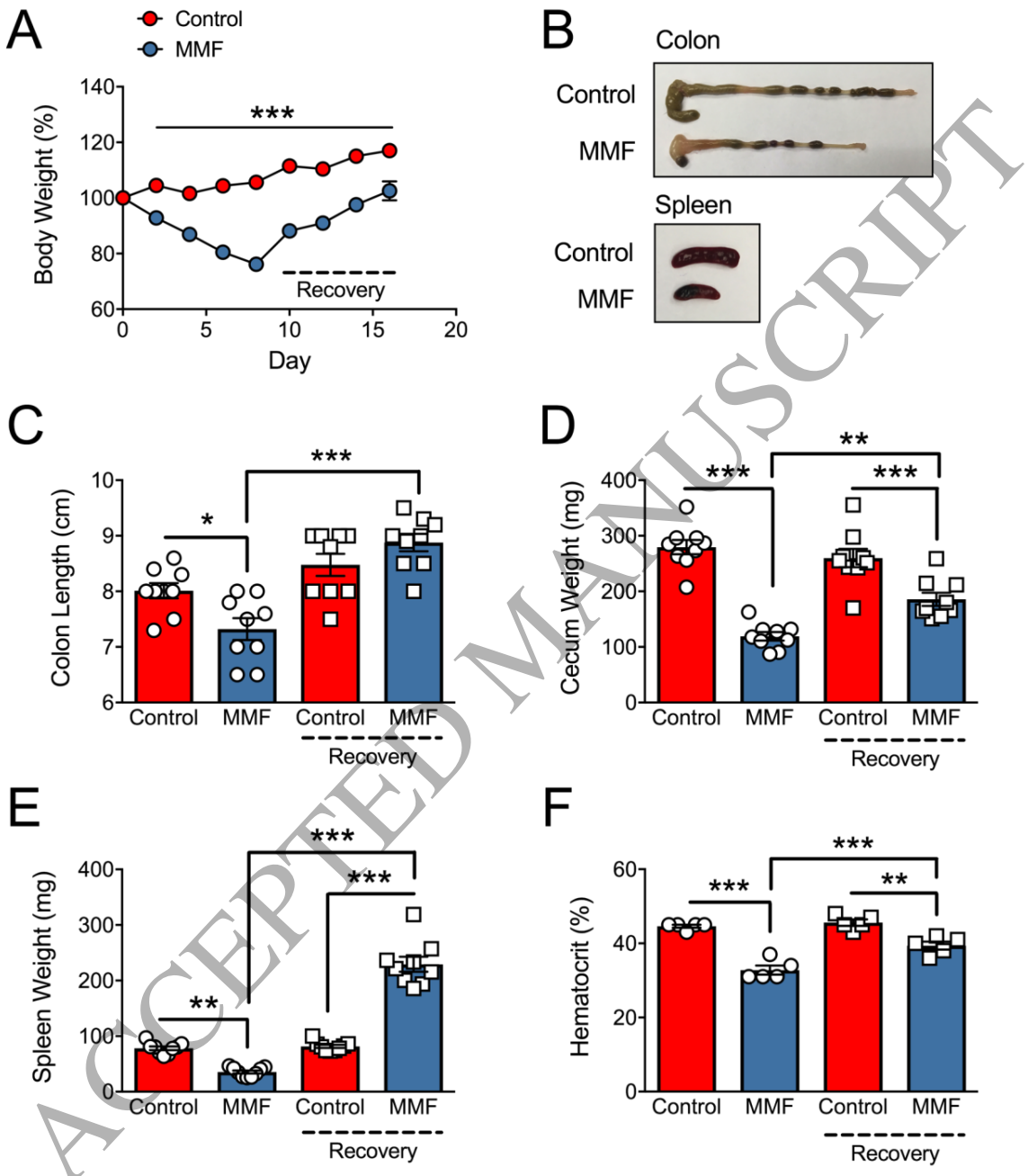

Figure 1. MMF exposure results in weight loss and gross structural changes in mouse colon. (A-E) Significant changes in body weight, colon length and spleen size, cecum weight, spleen weight and hematocrit in C57BL/6 mice after consuming chow containing 0.563% MMF *ad*

libitum for 8 days compared to control chow. On day 8 of the experimental period, the MMF chow group was switched to control feed and allowed to recover for 8 days. Data are pooled from two independent experiments with n=9 per group (except panel E; n=5 per group from one experiment). *P<0.05, **P<0.01, ***P<0.001., two-way ANOVA (A), one-way ANOVA (C-F).

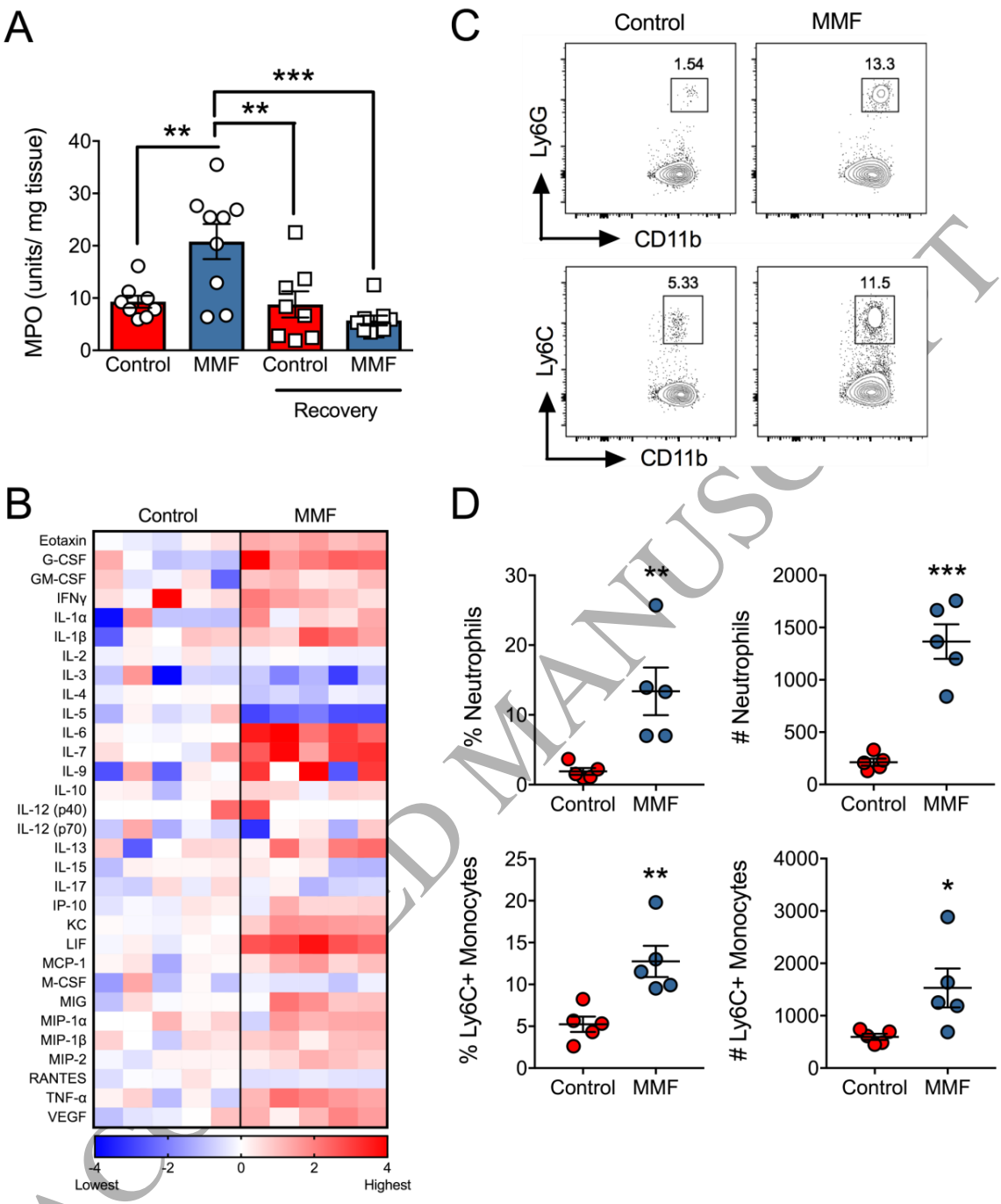

Figure 2. MMF exposure induces colonic inflammation. (A) Myeloperoxidase (MPO) activity in colon tissue was assessed after 8 days of MMF treatment and 8 days after MMF was withdrawn (Recovery). Results are pooled from two independent experiments with n=9 in each group. (B) Heat map indicating changes in colonic tissue cytokine profiles (protein) following 8 days of

MMF exposure vs. control animals (n=5). All values were normalized relative to control means and expressed as Log2 values (Blue indicates lowest expression and red indicates highest expression). (C) Representative fluorescence-activated cell sorting (FACS) plots with (D) cells counts indicating percentage and number of colonic neutrophils and inflammatory monocytes in control and MMF-fed mice. Data are representative of two independent experiments with n=5. *P<0.05, **P<0.01, ***P<0.001., one-way ANOVA. (A), Student t-test (D).

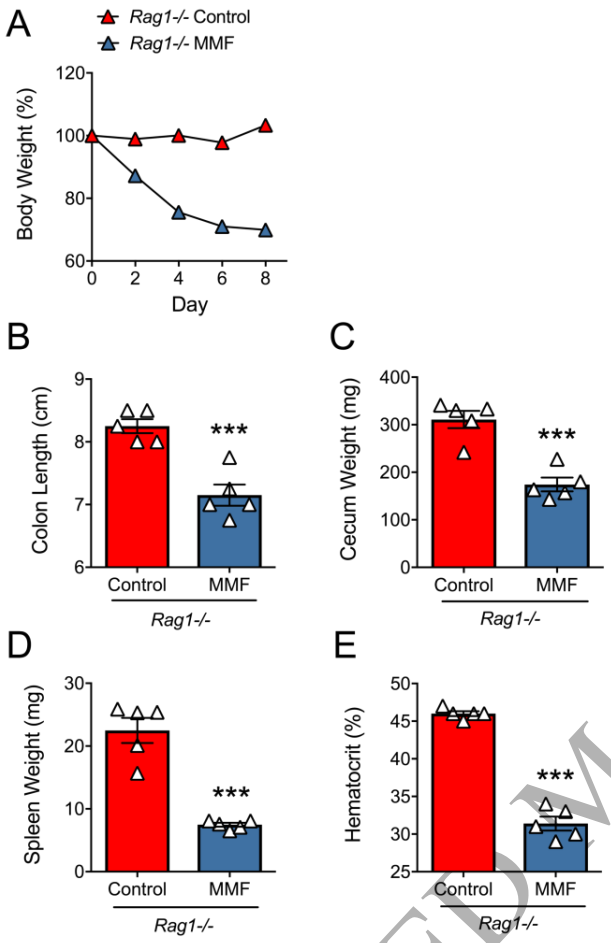

Figure 3. MMF exposure induces weight loss and colonic changes in *Rag1-/-* mice lacking adaptive immunity identical to wildtype animals. (A) Changes in body weight, (B) colon length, (C) cecum weight, (D) spleen weight, and (E) hematocrit in *Rag1-/-* mice fed control chow or MMF-containing chow for 8 days. Data are representative of two independent experiments with n=5 in each group. ***P<0.001, two-way ANOVA (A), Student t-test (B-E).

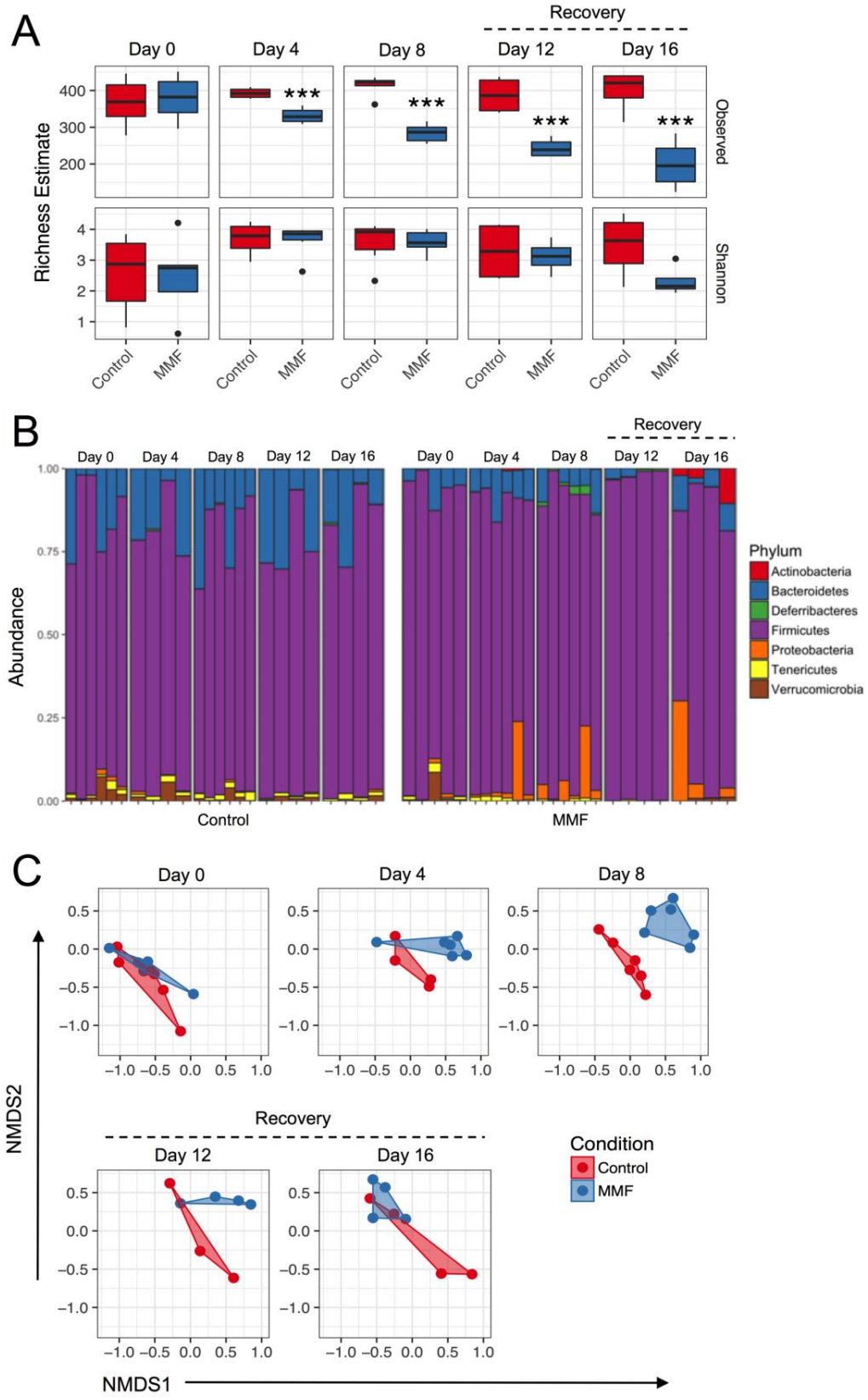
A
Recovery
Day 0
Day 4
Day 8
Day 12
Day 16
Richness Estimate
Observed
Shannon
***
Control
MMF
B
Recovery
Abundance
Control
MMF
Phylum
Actinobacteria
Bacteroidetes
Deferribacteres
Firmicutes
Proteobacteria
Tenericutes
Verrucomicrobia
C
NMDS1
NMDS2
Condition
Control
MMF

Figure 4. MMF exposure promotes changes in the diversity and composition of the intestinal microbiota. Data from 16S rRNA sequencing was utilized to determine changes in bacterial diversity and abundance during MMF exposure and following its withdrawal (Recovery). (A) α-diversity measurements (observed diversity and Shannon Diversity) of the fecal microbiota, (B) stacked histograms displaying alterations in intestinal bacterial phyla and (C) non-metric multidimensional scaling plot of β-diversity measurements using Bray-Curtis dissimilarity metric to indicate differences in microbial composition at different time points between control mice and mice exposed to MMF and during recovery (n=4-6; see Materials and Methods). ***P<0.001, two-way ANOVA.

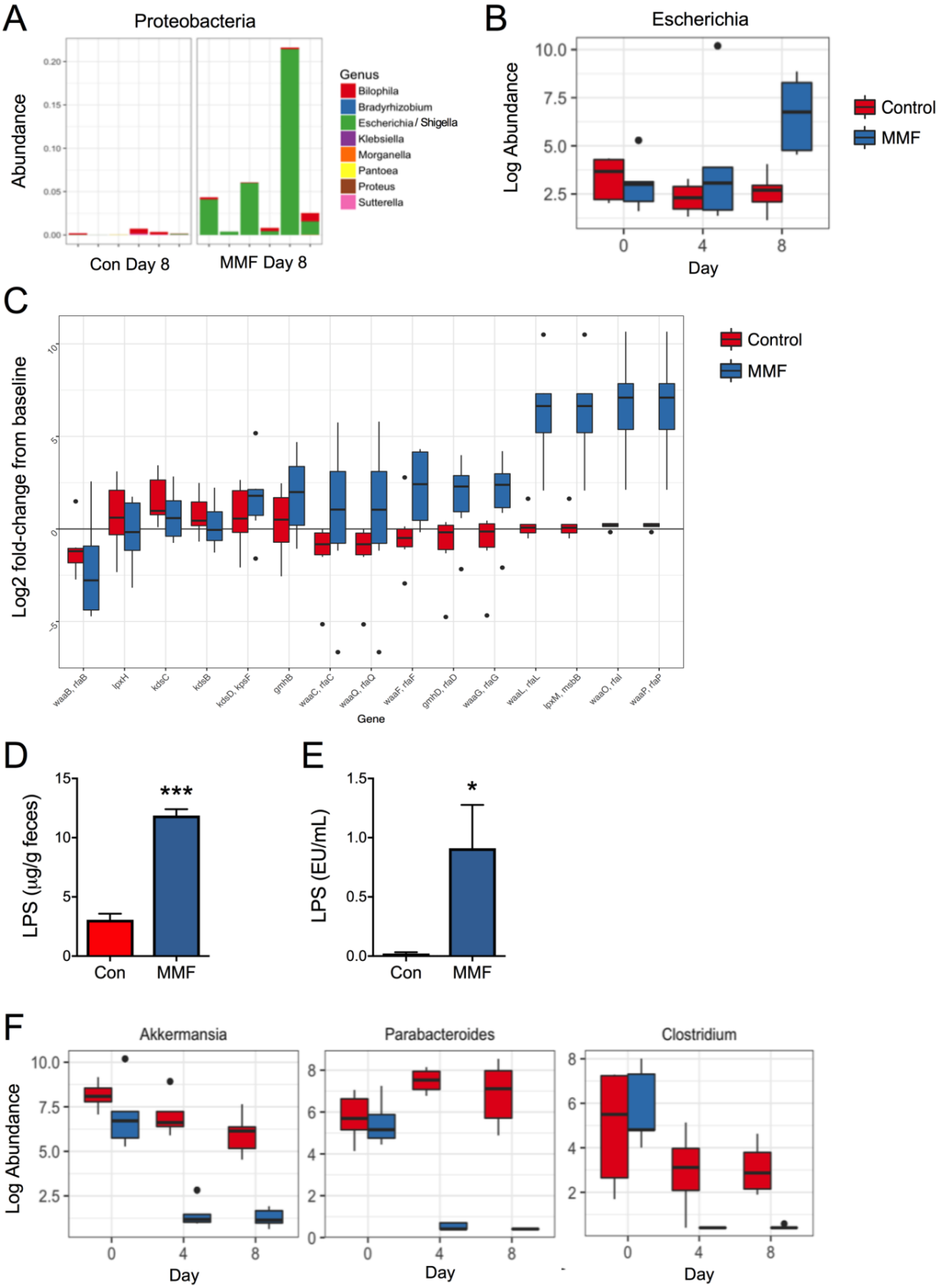
A
Proteobacteria
Abundance
0.20
0.15
0.10
0.05
0.00
Con Day 8
MMF Day 8
Genus
Bilophila
Bradyrhizobium
Escherichia / Shigella
Klebsiella
Morganella
Pantoea
Proteus
Sutterella
B
Escherichia
Log Abundance
10.0
7.5
5.0
2.5
0
4
8
Day
Control
MMF
C
Log2 fold-change from baseline
10
5
0
-5
waaB, rfaB
lpxH
kdsC
kdsB
kdsD, kpsF
gmhB
waaC, rfaC
waaQ, rfaQ
waaF, rfaF
gmhD, rfaD
waaG, rfaG
waaL, rfaL
lpxM, msbB
waaO, rfaI
waaP, rfaP
Gene
Control
MMF
D
LPS (μg/g feces)
15
10
5
0
***
Con
MMF
E
LPS (EU/mL)
1.5
1.0
0.5
0.0
*
Con
MMF
F
Akkermansia
Parabacteroides
Clostridium
Log Abundance
10.0
7.5
5.0
2.5
8
6
4
2
0
0
4
8
Day

Figure 5. MMF exposure promotes the overabundance of *Escherichia/Shigella*, increases microbial LPS levels, and results in the loss of key bacteria genera. (A) Analysis of the phylum Proteobacteria after 8 days of MMF exposure and (B) increasing abundance of the genus *Escherichia/Shigella* over 8 days of MMF exposure. (C) PICRUSt-predicted gene counts for the KEGG reference pathway for LPS biosynthesis showing genes that were enriched greater than log2 fold change. (D,E) LPS load in mouse feces and serum after 8 days of MMF administration. (F) Specific bacterial genera depleted in MMF-treated mice compared to control mice (n=4-6 per group). Data represent mean ± SEM. ***P<0.001, Student t-test (D,E).

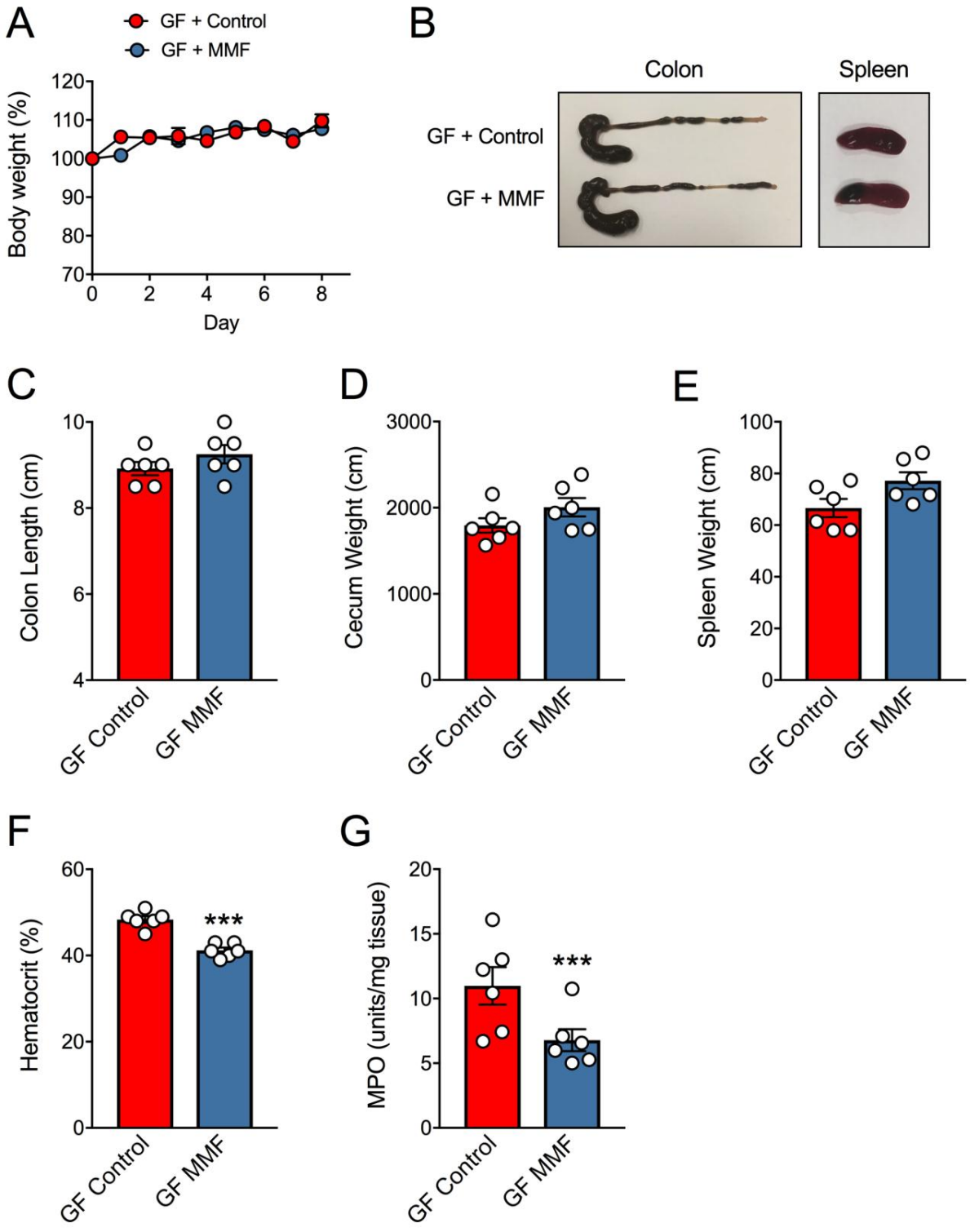

Figure 6. The intestinal microbiota is required to induce the toxic effects of MMF. Control or MMF-supplemented diets were fed to GF C57BL/6 mice in sterile conditions. (A-G) body weight,

colon length and spleen size, cecum weight, spleen weight, hematocrit and colonic MPO in GF mice exposed to MMF compared to GF mice on control diet (n=6 per group). Data represent mean ± SEM. ***P<0.001, Student's t-test (C-G).

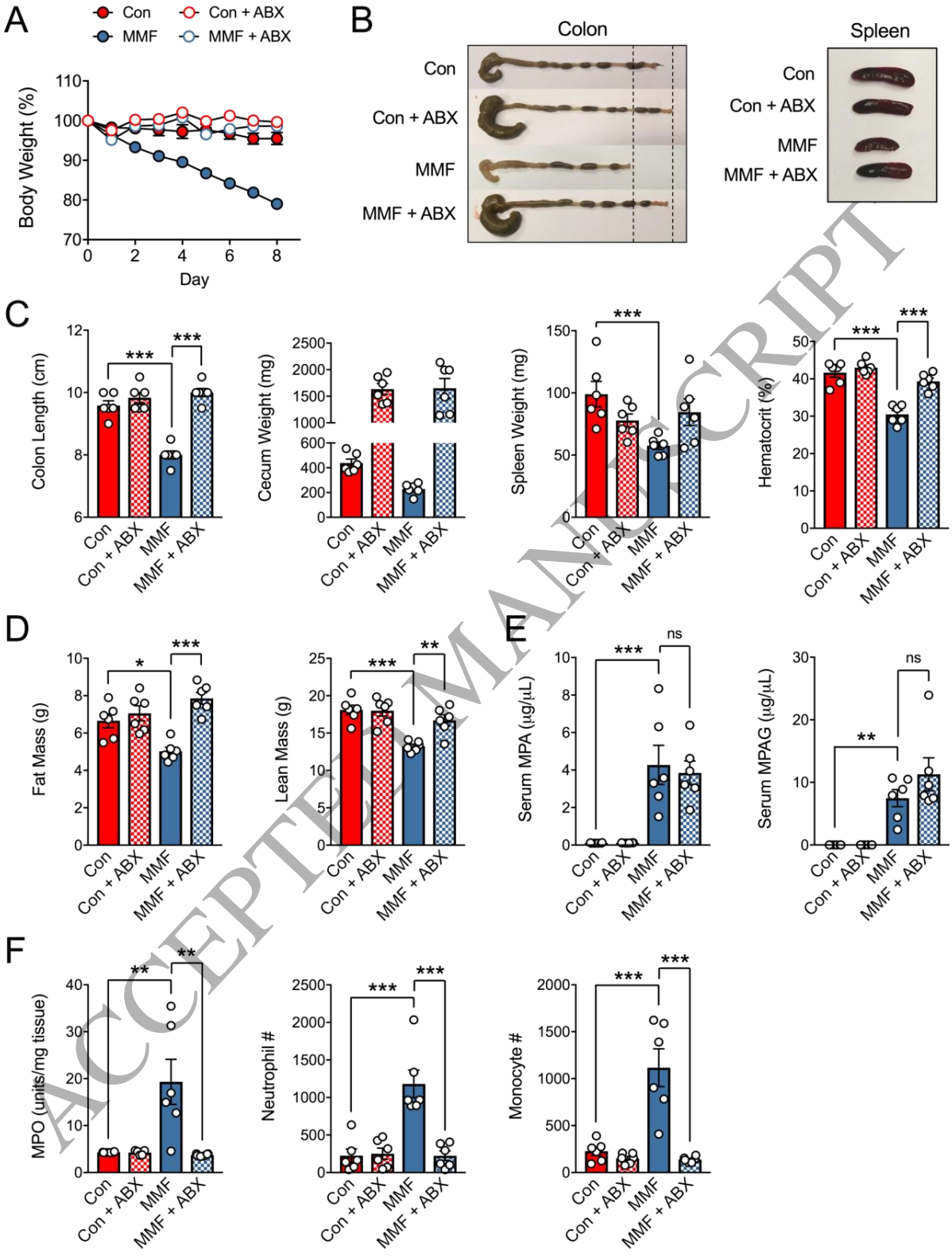

Figure 7. MMF toxicity is prevented by the administration of a cocktail of broad-spectrum antibiotics (ABX). Mice were treated with ABX or control water for 2 weeks followed by MMF administration for 8 days. (A-D) Effects of ABX exposure on body weight, colon length and spleen size, cecum weight, spleen weight, hematocrit and body composition after 8 days of MMF administration. (E) Serum levels of MPA and MPAG in mice after ABX treatment with or without MMF exposure. (F) Effect of ABX exposure on inflammatory indices in the colon following MMF exposure (n=6 per group). Data represent mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ns=not significant, two-way ANOVA (A), one-way ANOVA (D-F).