IMPACT OF ANTIBIOTIC-RESISTANT INTESTINAL BACTERIA ON MUCOSAL IMMUNE ACTIVATION AND ANTIMICROBIAL DEFENSES

by

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DEDICATION

I would like to dedicate this thesis to my family and loving partner: James W. Keith Sr., Laura Keith, Linda Keith Dunnavant, Anna Leigh Keith, John Keith, and Lisa Boscov-Ellen. Without their love and support over the years, none of this would have been possible.

ABSTRACT

Antibiotic treatment of patients undergoing complex medical treatments can deplete commensal bacterial strains from the intestinal microbiota, thereby reducing colonization resistance against a wide range of antibiotic-resistant pathogens. Expansion of antibiotic-resistant pathobionts in the intestinal lumen predisposes patients to bloodstream invasion and sepsis. The impact of intestinal domination by these antibiotic-resistant pathogens on mucosal immune defenses and epithelial and mucin-mediated barrier integrity is unclear. The studies presented here sought to examine both the impact of intestinal domination by antibiotic-resistant bacterial species and strains on the colonic mucosa, and also to examine the impact of monocytes on preventing bacterial dissemination from the intestine of mice densely colonized by *Klebsiella pneumoniae*.

To begin, we found that we could densely colonize the intestine of antibiotic-treated mice with a panel of different antibiotic-resistant clinical isolates, including Vancomycin-resistant *Enterococcus faecium* (VRE), *Klebsiella pneumoniae* (Kp), *Escherichia coli* (Ec), and *Proteus mirabilis* (Pm) at similar densities and doing this did not induce histologic evidence of epithelial damage. We observed variation in both intraluminal localization of bacterial strains as well as thickness of the dense mucin layer after dense colonization and determined it was influenced by which bacterial strain was colonized. Next, in order to test the hypothesis that the residual microbiota after antibiotic treatment can influence the severity of colitis caused by infection with *Clostridioides difficile*, we infected mice that were densely colonized with Kp, Pm, Ec, or others with a virulent strain of *C. difficile* and we found that the severity of *C. difficile* infection and mortality did not vary significantly between mice colonized with different antibiotic-resistant bacterial species. Our results suggest that

the virulence mechanisms enabling *C. difficile* infection (CDI) and epithelial destruction overwhelm the relatively minor impact of less virulent, antibiotic-resistant pathogens on the outcome of CDI.

In healthcare settings, Kp producing the KPC-type carbapenemase have emerged as a widespread cause of multidrug-resistant nosocomial infections, making these highly antibiotic resistant strains increasingly difficult to treat. There is currently minimal understanding of how Kp, despite not inducing overt inflammation, is able mechanistically to traverse the intestinal epithelial barrier and enter the bloodstream. Monocytes are bone-marrow residing leukocytes that can rapidly traffic into the bloodstream and circulate to infected or inflamed tissues. Rapid monocyte recovery following allo-HCT is correlated with improved overall survival, and our lab has previously found that monocytes play a crucial role in controlling lung Kp infections. Therefore, we hypothesized that monocytes could be playing a critical role in preventing Kp systemic dissemination from the intestine. We used a mouse model in which we could deplete the monocyte compartment in mice and determined that monocyte depletion leads to significant dissemination of Kp from the intestine of Kp dominated mice. Through the use of additional testing in several immunodeficient mouse backgrounds, we determined that Cybb ($gp91^{phox}$)$^{-/-}$ mice recapitulate the same phenotype, suggesting that monocytes' ability to perform intracellular killing on Kp can prevent or contain systemic dissemination from the intestine. Collectively, these findings provide an investigation into the host immune response to dense intestinal colonization of antibiotic resistant bacteria, highlighting the differences that manifest both when the intestinal epithelium is intact and when epithelial integrity is challenged.

BIOGRAPHICAL SKETCH

James was born to James W. Keith Sr. and Laura Keith on June 24th, 1988 in Memphis, TN. At the age of three his family moved to Moultrie, GA. James graduated from Colquitt County High School in 2006, and soon after moved to Poughkeepsie, NY to attend Vassar College, where he majored in Biology (BA). While at Vassar, James began his scientific training under the mentorship of Dr. Erica Crespi. During his time in the Crespi lab, James studied the role of leptin in the stress response of wood frogs. After graduation in the year 2010, James moved to Philadelphia to work in the lab of Dr. Kyong-Mi Chang at the University of Pennsylvania. At the University of Pennsylvania, James studied immune mechanisms of viral persistence and liver disease pathogenesis in human hepatitis B and C viruses. After two years, James entered his doctoral training at the Louis. V. Gerstner Jr. Graduate School of Biomedical Sciences at Memorial Sloan Kettering Cancer Center, where he worked under the mentorship of Dr. Eric Pamer.

ACKNOWLEDGEMENTS

I would first like to thank my thesis mentor, Dr. Eric Pamer, for all the time and effort he has invested into training me over the last five years. Throughout my time in graduate school he provided incredible mentorship and trusted me with large amounts of independence, which was critical to accomplishing the work presented here.

I would like to thank Ingrid Leiner and Drs. Simone Becattini and Mike Abt for providing important hands-on support and guidance throughout my training. Together they greatly aided in making me think more critically and creatively. Without Ingrid's help, truly nothing in the lab would be possible. Everyone else in the Pamer lab has also been invaluable in their own unique ways, and I cannot thank them enough for their support and friendship.

I would to thank my thesis committee members, Drs. Tobias Hohl and Alexander Rudensky, for their help in driving the project forward over the years. They both provided me with important critiques and experimental ideas throughout my training that helped me accomplish the work presented here.

Lastly, I would like to thank the entire graduate school, past members and present. Additionally, I have a tremendous debt of gratitude for the hard work and vision from Drs. Ken Marians, Harold Varmus, Tom Kelly and our benefactor Louis V. Gerstner Jr., who together established this school and helped cultivate the success that it has become today.

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INTRODUCTION TO THE THESIS

In recent decades, medical care has increasingly involved prevention and treatment of infections caused by antibiotic-resistant microbes that are acquired in healthcare settings. Some of these microbes have acquired resistance to all currently approved antibiotics and thus have become nearly untreatable. This has raised the possibility of a post-antibiotic era in which minor infections could frequently progress to death, as they did in the pre-antibiotic era.

The contribution of the microbiota to human health involves all organ systems, extending from the skin to the gastrointestinal tract and from hematopoietic organs to the central nervous system, with human and mouse studies identifying exciting and potentially clinically important correlations between microbiota composition and numerous diseases, and interest in exploiting the microbiota and the associated metabolome as a new approach to treating these diseases is rapidly increasing in academic, biotech, and pharmaceutical circles. My graduate work has largely focused on improving our understanding of the complex host-microbe interactions, both in how a bacterial pathogen interacts with the host and the host's indigenous microbiota during disease. With the use of *Clostridoides difficile*, we were able to examine multiple components of the tripartite interaction among the pathogen, the host, and the indigenous microbiota.

Chapter one summarizes a broad survey of the current scientific literature regarding complex relationships between commensal bacterial species, mammalian hosts and invasive pathogens, as well as the potential of clinically

important approaches to improve human health and resistance to infection. Chapter two examines the impact of dominating bacterial species on colonic immune defenses, and how pathogenetic mechanisms engaged by *C. difficile* to cause colitis and the host's immune defenses leading to resolution override the relatively minor contribution from the residual microbiota on the host immune response. Chapter three shows that monocytes and NADPH oxidase activity play an important role in preventing *Klebsiella pneumoniae* dissemination from the intestine. These findings reveal new insights into how bacterial pathogens interact with both the host immune system and the host's microbiota during disease.

MATERIALS AND METHODS

Mice

C57BL/6 (wild-type [WT] B6) mice were purchased from the Jackson Laboratory. Generation of CCR2-DTR mice was previously described (Hohl et al., 2009). *Cybb* (gp91^{phox}) $^{-/-}$, *Tnfa*$^{-/-}$, *Il17*$^{-/-}$, and *Rag*$^{-/-}$ were purchased from the Jackson Laboratory. Rag$\gamma c^{-/-}$ mice were purchased from Taconic Farms. *Il22*$^{-/-}$ mice were provided by R. Flavell (Yale University). All knockout mouse strains were derived on a C57BL/6 background. All mice were bred and maintained under specific pathogen-free conditions at the Memorial Sloan Kettering Research Animal Resource Center. Sex- and age-matched controls were used in all experiments according to institutional guidelines for animal care. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Memorial Sloan Kettering Cancer Center.

Bacterial growth conditions and quantification of bacterial burden

Clinical isolates were obtained from the Clinical Microbiology Laboratory and derived from blood cultures from patients undergoing treatment at Memorial Hospital, Memorial Sloan Kettering Cancer Center. All the bacteria were grown in LB medium with shaking at 37°C. Bacterial burden was quantified by plating serial dilutions of homogenized organs on LB plates supplemented with the appropriate antibiotics.

Antibiotic Pretreatment, Ampicillin resistant test strain administration, CDI, and Mouse Monitoring

Mice were cohoused for 2 weeks prior to antibiotic treatment and then treated

with ampicillin (Sigma) (0.5 g/l). After four days, mice were administered 20,000 CFUs of an ampicillin-resistant test strain grown in LB broth supplemented with ampicillin. Two days later, ampicillin was withdrawn from drinking water. Twenty-four hours post ampicillin cessation, mice received
200 *C. difficile* spores (VPI10463 strain ATCC #43255) via oral gavage. After infection, mice were monitored and scored for disease severity by four parameters weight loss (> 95% of initial weight = 0, 95%–90% initial weight = 1, 90%–80% initial weight = 2, < 80% = 3), surface body temperature (> 32°C = 0, 32°C–30°C = 1, 30°C–28°C = 2, < 28°C = 3), diarrhea severity (formed pellets = 0, loose pellets = 0, liquid discharge = 2, no pellets/caked to fur = 3), morbidity (score of 1 for each symptoms with max score of 3; ruffled fur, hunched back, lethargy, ocular discharge).

Flow Cytometry

Single-cell suspensions were stained and analyzed on a BD LSR II cytometer. Antibodies were purchased from BD Bioscience. For monocyte and neutrophil staining the following antibodies were used: anti-Ly6C (clone AL-21), Ly6G (1A8), CD11b (M1/70), CD45 (30F-11), CD11c (HL3), CD103 (2E7). Cell viability was assessed with Live/Dead AQUA stain (Invitrogen). All flow cytometry data was analyzed by FlowJo v 9.7 (Treestar).

Histology Sectional and Pathology Scoring

Colon tissues were fixed with 4% paraformaldehyde and embedded in paraffin, and 5 μm sections were cut and stained with hematoxylin and eosin. H&E-stained colon tissue sections were blindly scored as described (Jarchum et al.,

2012) based on epithelial degeneration/cell death, edema, and cellular infiltration, with each parameter scored from 0 to 3.

Quantification of C. difficile burden and toxin

Fecal pellets or cecal content were resuspended in deoxygenated phosphate-buffed saline (PBS), and ten fold dilutions were plated on BHI agar supplemented with yeast extract, taurocholate, L-cysteine, cycloserine and cefoxitin at 37°C in an anaerobic chamber (Coylabs) overnight. (Sorg and Dineen, 2009). The presence of *C. difficile* toxins was determined using a cell-based cytotoxicity assay as previously described (Jarchum et al., 2011). Briefly, human embryonic lung fibroblast WI-38 cells (ATCC# CCL-75) were incubated in a 96-well plate overnight at 37°C. Ten fold dilutions of supernatant from resuspended cecal content was added to WI-38 cells, incubated overnight at 37°C and the presence of cell rounding and death was observed the next day. The presence of *C. difficile* toxins A and B was confirmed by neutralization by antitoxin antisera (Techlab, Blacksburg, VA). The data are expressed as the $\log_{10}$ reciprocal value of the last dilution where cell rounding was observed.

DNA extraction and 16S sequencing

DNA extraction from fecal pellets and intestinal content were performed as previously described (Ubeda et al., 2012). In brief, a frozen aliquot (~100 mg) of each sample was suspended, while frozen, in a solution containing 500 µl of extraction buffer (200 mM Tris, pH 8.0, 200 mM NaCl, and 20 mM EDTA), 200 µl of 20% SDS, 500 µl of phenol/chloroform/isoamyl alcohol (24:24:1), and 500 µl of

0.1-mm-diam zirconia/silica beads (BioSpec Products). Microbial cells were lysed by mechanical disruption with a bead beater (BioSpec Products) for 2 min, after which two rounds of phenol/chloroform/isoamyl alcohol extraction were performed. DNA was precipitated with ethanol and resuspended in 50 µl of TE buffer with 100 µg ml−1 RNase. The isolated DNA was subjected to additional purification with QIAamp Mini Spin Columns (QIAGEN). For each sample, duplicate 50 µl PCR reactions were performed, each containing 50 ng of purified DNA, 0.2 mM dNTPs, 1.5 mM MgCl2, 2.5 U Platinum Taq DNA polymerase, 2.5 µl of 10X PCR buffer, and 0.5 µM of each primer designed to amplify the V4-V5: 563F (5′-nnnnnnnn-NNNNNNNNNNNN-AYTGGGYDTAAAGNG-3′) and 926R (5′-nnnnnnnn-NNNNNNNNNNNN-CCGTCAATTYHTTTRAGT-3′). A unique 12-base Golay barcodes (Ns) precede the primers for sample identification (Caporaso et al., 2012), and 1–8 additional nucleotides were placed in front of the barcode to offset the sequencing of the primers. Cycling conditions were 94°C for 3 min, followed by 27 cycles of 94°C for 50 s, 51°C for 30 s, and 72°C for 1 min. 72°C for 5 min is used for the final elongation step. Replicate PCRs were pooled, and amplicons were purified using the Qiaquick PCR Purification kit (QIAGEN). PCR products were quantified and pooled at equimolar amounts before Illumina barcodes and adaptors were ligated on using the Illumina TruSeq Sample Preparation protocol. The completed library was sequenced on an Illumina Miseq platform following the Illumina recommended procedures with a paired end 250 × 250 bp kit. The 16S (V4-V5) paired-end reads were merged and demultiplexed. The UPARSE pipeline (Edgar, 2013) was used to: (1)

perform error filtering, using maximum expected error (Emax = 1; Edgar and Flyvbjerg, 2015); (2) group sequences into operational taxonomic units (OTUs) of 97% distance-based similarity; and (3) identify and remove potential chimeric sequences, using both de novo and reference-based methods. Sequencing data were analyzed and processed using the MOTHUR pipeline (Schloss et al., 2009), and operational taxonomical units (OTU) were classified using a modified version of the Greengenes database (DeSantis et al., 2006).

Tissue RNA isolation, cDNA preparation and RT-PCR

RNA was isolated from colon tissue using mechanical homogenization and TRIzol isolation (Invitrogen) according to the manufacturer's instructions. cDNA was generated using QuantiTect reverse transcriptase (Qiagen). RT-PCR was performed on cDNA using Taqman primers and probes in combination with Taqman PCR Master Mix (ABI) and reactions were run on a RT-PCR system (Step-one Plus; Applied Biosystems). Gene expression is displayed as fold increase over uninfected C57BL/6 mice and normalized to *Hprt*.

Fluorescence *in-situ* hybridization (FISH)

The hybridization method was adapted from Swidsinski et al., 2005 and Vaishnava at el., 2011. Briefly, tissue sections were deparaffinized with xylene (twice, 10 min each) and rehydrated through an ethanol gradient (95%, 10 min; 90%, 10 min) to water. Sections were incubated with a universal bacterial probe directed against the 16S rRNA gene at 50°C for 3 hours. Probes were diluted to 5ng/µl in 0.9M NaCl, 20mM Tris-HCl at pH7.2 and 0.1% SDS prior to use.

Sections were later washed twice in 0.9M NaCl, 20mM Tris-HCl at pH7.2 (wash buffer) for 10 min and counterstained with Hoechst (1:3000 in wash buffer) for nuclear staining as well as a cocktail of Fluorescein conjugated Dolichos Biflorus Agglutinin, Soybean Agglutinin, Sambucus Nigra Bark Lectin, Ulex Europaeus Agglutinin I, and Wheat Germ Agglutinin (Vector Labs) (1:50 dilution in wash buffer). The FISH probe used was universal bacterial probe EUB338: [Cy3]-GCTGCCTCCCGTAGGAGT-[AmC7~Q+Cy3es].

Microscopy

Images were acquired with a Leica TCS SP5-II upright confocal microscope using a 20x lens (NA 1.4, HCX PL APO) as a series of short Z-stacks. Maximum intensity projection processing of Z-stacks was done in Fiji (ImageJ) software. Mucus layer thickness was measured using the Leica distance measurement tool (LASAF). Whole tissue images were digitally scanned using the Zeiss Mirax Desk Scanner with 20x/0.8NA objective. Bacterial distance analysis was performed on colon images taken using the Zeiss Mirax Desk Scanner with 20x/0.8NA objective by determining the XY coordinates of each bacterial cell in MetaMorph (Molecular Devices) software and measuring the distance from their center. For quantification of bacterial density and invasion into the mucus layer, whole tissue cross-sections were tile scanned in short Z-stacks using an inverted laser scanning confocal Zeiss LSM 5-Live microscope at 63x magnification. For bacterial quantification, a threshold based on the RGB color combination and intensity of each bacterial species was generated with the color thresholding

option in MetaMorph. Thresholded objects of 1μm in size were counted as a single bacterial cell with MetaMorph's integrated morphometric analysis tool.

Statistical Analysis

Results represent means ± SEM. Statistical significance was determined by the unpaired t test, Mann-Whitney test for $n \leq 5$, two-way ANOVA test for time course experiments, and log-rank test for survival curve. Statistical analyses were performed using Prism GraphPad software v6.0 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

CHAPTER 1

ENLISTING COMMENSAL MICROBES TO RESIST ANTIBIOTIC-RESISTANT PATHOGENS[1]

1.1 Abstract

The emergence of antibiotic-resistant bacterial pathogens is an all-too-common consequence of antibiotic use. Although antibiotic resistance among virulent bacterial pathogens is a growing concern, the highest levels of antibiotic resistance occur among less pathogenic but more common bacteria that are prevalent in healthcare settings. Patient-to-patient transmission of these antibiotic resistant bacteria is a perpetual concern in hospitals. Many of these resistant microbes, such as vancomycin-resistant *Enterococcus faecium* and carbapenem-resistant *Klebsiella pneumoniae*, emerge from the intestinal lumen and invade the bloodstream of vulnerable patients, causing disseminated infection. These infections are associated with preceding antibiotic administration, which changes the intestinal microbiota and compromises resistance to colonization by antibiotic-resistant bacteria. Recent and ongoing studies are increasingly defining commensal bacterial species and the inhibitory mechanisms they use to prevent infection. Use of next-generation probiotics derived from the intestinal microbiota represents an alternative approach to prevention of infection by enriching colonization with protective commensal species, thereby reducing the density of antibiotic resistant bacteria and also reducing patient-to-patient transmission of

[1] Chapter 1 summarizes the work published in Keith, J.W., and E.G. Pamer. 2019. Enlisting commensal microbes to resist antibiotic-resistant pathogens. *J Exp Med* 216:10-19.

infection in healthcare settings.

1.2 Introduction

Over the past three decades, medical care has increasingly involved prevention and treatment of infections caused by antibiotic-resistant microbes that are acquired in healthcare settings. Some of these microbes have acquired resistance to all currently approved antibiotics and thus have become essentially untreatable. This has raised the specter of a post-antibiotic era in which minor infections could frequently progress to death, as they did in the pre-antibiotic era. Modern-day humans have mostly lived in an era of readily-treated bacterial infections, and the concept of dying from a minor cut or scratch is foreign to most of us.

The contribution of the microbiota to human health involves all organ systems, extending from the skin to the gastrointestinal tract and from hematopoietic organs to the central nervous system (Belkaid and Segre, 2014; Caballero and Pamer, 2015; Manzo and Bhatt, 2015; Sharon et al., 2016). Human and mouse studies have identified exciting and potentially clinically important correlations between microbiota composition and diseases such as obesity (Turnbaugh et al., 2009), liver disease (Henao-Mejia et al., 2013), malnutrition (Smith et al., 2013a), inflammatory bowel disease (Wlodarska et al., 2015), hypertension (Wilck et al., 2017), rheumatoid arthritis (Scher et al., 2013), cancer (Zitvogel et al., 2017), autism (Sharon et al., 2016) and Parkinson's disease (Sampson et al., 2016), and interest in exploiting the microbiota and the associated metabolome as a new approach to treating these diseases is rapidly

increasing in academic, biotech and pharmaceutical circles. The impact of microbiota composition on metabolic, inflammatory, autoimmune and neurologic diseases is readily measurable, statistically significant and, in some cases, sufficiently impressive to warrant clinical study. The effect of the microbiota on resistance to enteric infections is measured on a log scale, with susceptibility to certain infections reduced by a million-fold in the presence of a diverse microbiota. The development of next-generation probiotics derived from the commensal microbiota to reduce infections (Pamer, 2016), particularly those caused by antibiotic-resistant bacteria acquired in healthcare settings, represents the most straightforward, though arguably not the most glamorous, therapeutic target for clinical exploitation of the microbiota. However, moving from the experimental demonstration of a commensal bacterium's ability to enhance resistance against a pathogen to the development of a therapeutic probiotic will take time and extensive clinical study.

1.3 Commensal microorganisms & mechanisms of colonization resistance

Although Metchnikov speculated over 100 years ago that certain bacterial species constituting the microbiota contribute to disease resistance and human longevity (Brown and Valiere, 2004), the role of the microbiota in resistance to infectious diseases was not fully appreciated until potent antibiotics were introduced into medical practice in the 1940s. Clinicians caring for patients treated with penicillin or streptomycin noted that the bacterial populations colonizing their patients were altered by antibiotic treatment, leading to infections with yeasts and antibiotic-resistant bacteria (Keefer, 1951; Lipman et al., 1948;

Smith, 1952; Woods et al., 1951). These observations led to experimental studies with rodents in the 1950's by Miller, Bohnhoff and Freter that demonstrated marked increases in susceptibility to infection by Salmonella enteritidis (Miller et al., 1956), Shigella flexneri and Vibrio cholerae (Freter, 1956) following antibiotic treatment. Classical microbiologic studies led to the conclusion that obligate anaerobic commensal bacterial species were the most consequential contributors to resistance against S. enteritidis infection (Bohnhoff et al., 1964a). Subsequent studies demonstrated that autochthonous or exogenous Enterobacteriaceae undergo marked expansion in the GI tract of rodents and humans following antibiotic treatment, and the term "colonization resistance" was coined to denote the microbiota's capacity to inhibit expansion of Enterococci and Enterobacteriaceae in the gut lumen (Clasener et al., 1987; Van der Leur et al., 1993).

Since the advent of microscopy over three centuries ago and the description of pleomorphic "animalcules" that reside in the mouth (Lane, 2015), it has been understood that our surfaces, particularly along the gastrointestinal tract, are colonized with dense and diverse populations of microbes. The complexity of organisms inhabiting our colons was demonstrated by deep sequencing of highly variable regions of bacterial 16S rRNA genes from fecal samples, allowing for generation of phylogenetic trees. Sequencing of over 13,000 16S rRNA genes from the colons of three healthy individuals demonstrated that humans harbor highly diverse bacterial populations, with dramatic person-to-person variation in microbiota composition (Eckburg et al.,

2005). The Human Microbiome Project and the MetaHit Program used next generation sequencing platforms to characterize the microbiota of hundreds of healthy individuals, confirming the substantial interindividual variation (Arumugam et al., 2011; Human Microbiome Project, 2012). A consistent message from studies spanning a wide range of human populations is that, at baseline, the adult colonic microbiota comprises predominantly bacteria belonging to the Bacteroidetes or Firmicutes phyla (Fig 1A). These phyla contain many different families, genera and species of bacteria that vary in proportion between individuals but that remain remarkably constant within individuals in the absence of intestinal infection, dietary change or antibiotic administration (David et al., 2014).

1.4 Mechanisms of colonization resistance

The bacterial species constituting the colonic microbiota provide colonization resistance via a multitude of parallel mechanisms that restrict the ability of exogenous bacterial strains to gain a foothold in the gut, thereby reducing the host's susceptibility to enteric infections (Buffie and Pamer, 2013). Direct colonization resistance restricts engraftment of exogenous microbes and limits overly robust expansion of indigenous microbes, without enlisting host defenses. The major mechanisms of direct colonization resistance include bacterial competition for nutrients, direct antagonism/killing, and the production of inhibitory metabolites. Commensal bacteria derive their nutrients almost exclusively from dietary and host-derived carbohydrates, the abundance of which shapes the composition of the microbiota because bacterial strains differ in their

ability to utilize different carbohydrates (Walker et al., 2011); (David et al., 2014); (Martinez et al., 2013). Competition between commensal species is best characterized for bacteria belonging to the Bacteroidetes phylum. *Bacteroides fragilis* strains encode polysaccharide utilization loci (PUL) that enable them to deprive competing *B. fragilis* strains of required nutrients and thereby maintain long-term colonization (Lee et al., 2013). *Bacteroides ovatus* and *B. thetaiotaomicron* encode and transcribe distinct PULs that endow each with the ability to metabolize distinct carbohydrates (Martens et al., 2011), with reciprocal glycan preferences enabling both species to co-inhabit a complex ecosystem by occupying distinct metabolic niches. (Tuncil et al., 2017). While many bacterial species of the microbiota compete at the metabolic level, there are also examples of inter-species cooperation that facilitates carbohydrate metabolism, such as occurs when *Bacteroides ovatus*'s ability to digest extracellular polysaccharides benefits *Bacteroides vulgatus* (Rakoff-Nahoum et al., 2016). Dietary changes, such as reduced intake of fiber, can result in enhanced utilization of mucin-associated carbohydrates by Bacteroides species, which thins the protective inner mucin layer and reduces host resistance to infection (Desai et al., 2016) (Fig 1B). PULs within the Firmicutes phylum are distinct from those encoded by the Bacteroidetes, and the diversity of the Firmicutes PULs underlies their nutritional specialization and explains the fluctuations in representation of different bacterial taxa following changes in dietary fiber intake (Sheridan et al., 2016).

Commensal bacteria also produce bacteriocins—microbial products that

inhibit other bacteria but to which the producing bacteria are immune. These antimicrobial products can influence the stability and composition of complex microbial populations. For example, *Lactobacillus salivarius* produces a bacteriocin that inhibits *Listeria monocytogenes* (Corr et al., 2007), and Enterococci express bacteriocins that confer competitive advantages in the intestinal tract (Kommineni et al., 2015).. The human-derived commensal *Bacillus thuringiensis* produces a bacteriocin that inhibits spore-forming Gram-positive bacteria, including *Clostridium difficile*, while leaving the commensal microbiota composition intact (Rea et al., 2010); (Rea et al., 2011). *E. coli* Nissle 1917 is a probiotic that produces bacteriocins that reduce colonization by Gram-negative pathogens including *E. coli* and *Salmonella enterica* (Vassiliadis et al., 2010); (Sassone-Corsi et al., 2016).

Gram-negative commensals likely also mediate colonization resistance via the Type VI secretion systems (T6SSs), a mechanism of bacterial antagonism that involves direct, contact-dependent transport of antimicrobial toxins from donor to recipient bacteria via needle like structures (Russell et al., 2014); (Russell et al., 2011). T6SSs are common in Gram-negative commensals, with more than half of human intestinal Bacteroidales genomes and more than a quarter of all Proteobacterial genomes possessing T6SS genes (Boyer et al., 2009); (Coyne et al., 2016). The T6SS loci of human-derived Bacteroidales species segregate into three genetic architectures (GA), denoted as GA1, GA2, and GA3. Whereas GA1 and GA2 are shared among diverse human-derived Bacteroidales species, GA3 T6SSs are limited to *Bacteroides fragilis* and do not

transfer proteins to other Bacteroidales species. In vitro, T6SSs target many human gut-derived Bacteroidales strains lacking protective cognate immunity proteins (proteins produced alongside toxic effector proteins that shield the producing cell from toxicity), but they fail to inhibit E. coli (Chatzidaki-Livanis et al., 2016). Thus, T6SSs weaponize the competition between indigenous species, enabling some strains to persist in their niche by restricting invasion by exogenous species and limiting expansion of local competitors via direct killing (Chatzidaki-Livanis et al., 2016).

Microbial metabolic products, such as short chain fatty acids (SCFAs), also contribute to colonization resistance. The Firmicutes phylum encompasses a wide range of bacterial species that includes facultative anaerobes such as Lactobacillus and spore forming obligate anaerobes such as the Clostridia. Given their prevalence in the colonic microbiota, it is not surprising that these bacterial classes are major contributors to the overall metabolism of the lower gastrointestinal tract. It is now appreciated that bacteria belonging to the Lachnospiraceae and Ruminococcaceae families are the major producers of butyrate in the lower gastrointestinal tract (Barcenilla et al., 2000; Louis and Flint, 2009), thereby impacting colonic health, immune system development and colonization resistance. Butyrate production by commensal bacteria influences host mucosal immune development by promoting differentiation of regulatory T cells (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013b) and likely contributes to colonization resistance against enteric pathogens. A small subset of colonic Firmicutes, represented by *Clostridium scindens*, encode operons that

modify primary bile acids in the lower intestinal tract, generating secondary bile salts (Ridlon et al., 2006), which can enhance resistance against Clostridium difficile infection (Buffie et al., 2015). *Ruminococcus gnavus* converts the secondary bile acid deoxycholic acid to a less cytotoxic iso- bile acid that allows for preferential growth of some Bacteroides species, thereby potentially contributing to colonization resistance (Devlin and Fischbach, 2015) (Fig 1A).

1.5 Antibiotic-induced changes to the microbiota

Antibiotic treatment, while often remarkably effective at curing bacterial infections, can cause collateral damage to the patient's microbiota and markedly reduce resistance to colonization and infection by pathogens. Classic studies of the 1940s and 50s demonstrated the occurrence of antibiotic-induced changes in the microbiota, and next-generation sequencing has since provided a more comprehensive picture of the impact of antibiotics on the microbiota (Dethlefsen et al., 2008); (Dethlefsen and Relman, 2011), the extent of which often extends beyond their antibacterial spectra. For example, vancomycin, an antibiotic that interferes with bacterial cell wall synthesis, exclusively kills Gram-positive bacteria in vitro but also markedly reduces the prevalence of Gram-negative Bacteroidetes in vivo (Isaac et al., 2017; Ubeda et al., 2010). Other antibiotics, such as clindamycin and metronidazole, have broad, detrimental effects on microbiota composition in the mouse gut (Buffie et al., 2012; Lewis et al., 2015). Given the interdependencies of bacterial species in the microbiota, it is possible that direct elimination of antibiotic-sensitive bacterial species leads to indirect loss of dependent, albeit antibiotic-resistant, species (Fig 1C). Our knowledge of

the impact of antibiotics on the commensal microbiota, however, is far from complete and we are likely to learn much from longitudinal clinical studies of microbiota changes following initiation and completion of specific antibiotic treatments.

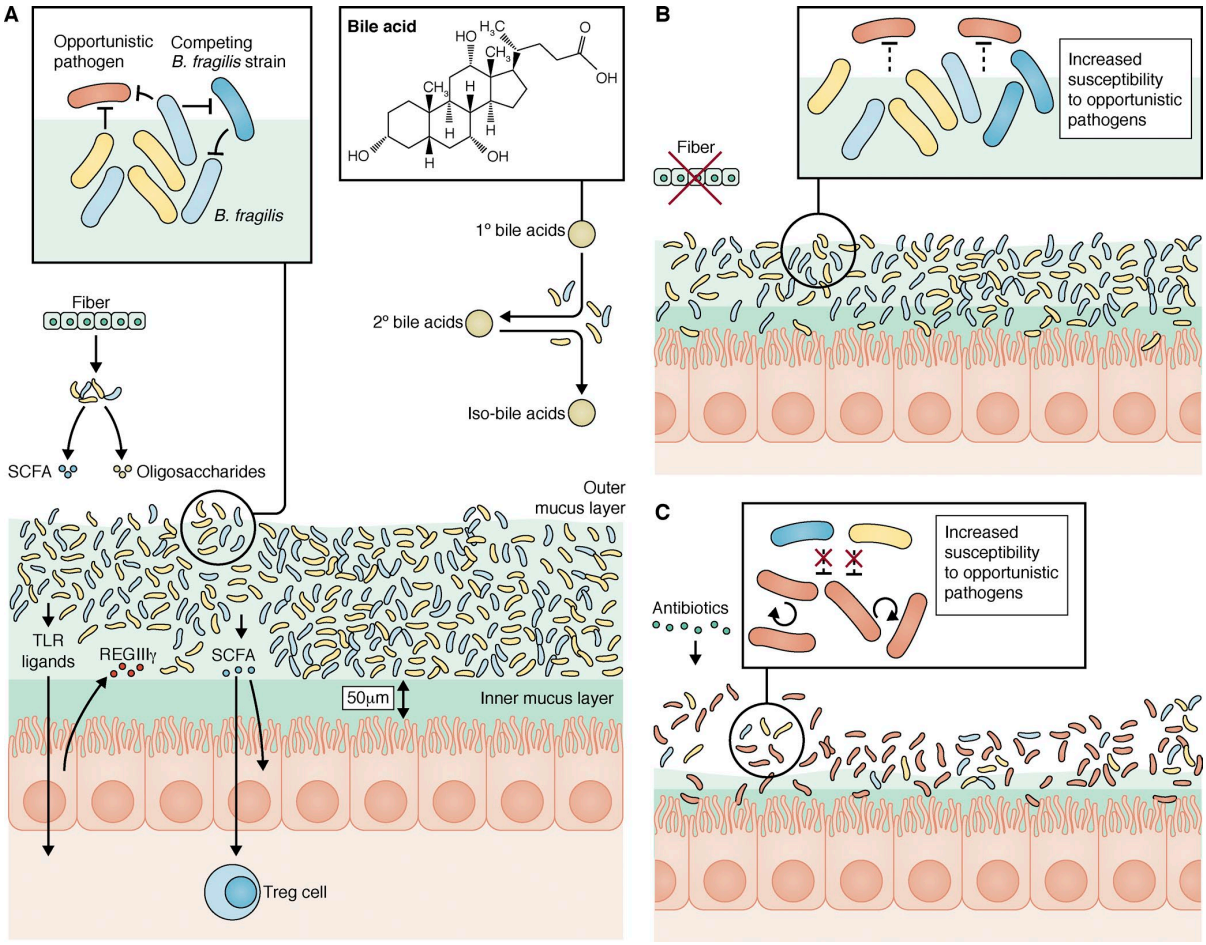

Figure 1: The microbiota plays an important role in intestinal homeostasis and prevention of opportunistic pathogen infection

A healthy microbiota is comprised predominantly of bacteria that are members of the Bacteroidetes (blue) and Firmicutes (yellow) phyla. These bacteria interact and cooperate to break down dietary fiber and host derived mucus into a variety of carbohydrates that support the complex community. Short chain fatty acids (SCFA) are a byproduct of carbohydrate fermentation that promote differentiation of regulatory T cells (Treg). Bacterial derived TLR ligands promote production of antimicrobial peptides such as Regenerating islet-derived protein IIIγ (RegIIIγ), helping prevent bacterial penetration into the inner mucus layer. Specific

bacterial species can produce secondary and Iso- bile acids, which contribute to colonization resistance against Clostridium difficile. Bacteria such as *B. fragilis* maintain long-term colonization by utilizing distinct polysaccharides so that similar strains that would otherwise use these same polysaccharides cannot engraft due to competitive exclusion. A healthy microbiota also allows for the maintenance of two distinct mucus layers- a ~50 micrometer epithelium-associated inner mucus layer that is largely impenetrable by intestinal bacteria and a less dense outer layer that serves as a microbial habitat. (A) Dietary fiber is an important substrate of the healthy microbiota, but when dietary changes result in low fiber availability, bacteria resort to using the glycoprotein-rich mucus layer as an alternative energy source. As a result, dietary changes can lead to thinning of the mucus layer, permitting increased bacterial penetration of the mucus layer, which can lead to epithelial inflammation and increased pathogen susceptibility. (B) Antibiotic administration disrupts complex feedback loops that sustain the complex microbial community, causing loss of mucus due to the diminishment of microbiota-derived host factors that regulate the production and secretion of mucus. In addition, some antibiotics can cause colonization resistance to be lost, leaving the host vulnerable to opportunistic enteric pathogen (red) expansion.

1.6 Pathogens of the healthcare environment

Although intestinal infections with bacterial pathogens such as *S. enteritidis*, *Shigella flexneri* and *Vibrio cholerae* remain major threats to human health, particularly in settings with limited resources, infections caused by less pathogenic but more antibiotic-resistant bacterial species have become an increasing problem in the developed world. Indeed, a recent Centers for Disease Control and Prevention publication listed the most threatening antibiotic-resistant pathogens (https://www.cdc.gov/drugresistance/biggest_threats.html), many of which are acquired in healthcare settings and can become problematic when the host's microbiota is dysregulated, most often by antibiotic administration itself. In the following sections, we will discuss the role of the intestinal microbiota in defense against these hospital-acquired pathogens and describe experimental studies and clinical trials that are revealing new approaches to reducing the risk of infection with and transmission of antibiotic-resistant bacteria. Finally, we

propose that reconstitution of the microbiota following broad-spectrum antibiotic treatment should become a routine part of medical practice.

1.7 Enterococcus faecalis and Enterococcus faecium

Enterococci are common commensal bacteria that colonize the intestine of nearly all terrestrial animals (Lebreton et al., 2017). *E. faecalis* and *E. faecium*, the main enterococcal species inhabiting the human gut, are non-pathogenic in the gastrointestinal tract but cause severe infections if they enter the bloodstream; such infections are challenging to treat because of antibiotic resistance (Arias and Murray, 2012). Vancomycin resistant *E. faecium* (VRE), for example, is one of the most common causes of bloodstream infection in patients undergoing treatment for leukemia or following bone marrow transplantation (Kamboj et al., 2010)and recent studies have demonstrated that the intestinal microbiota becomes dominated by VRE prior to invasion of the bloodstream (Taur et al., 2012; Ubeda et al., 2010). Antibiotics that kill obligate anaerobic bacteria of the colon predispose patients to dense intestinal colonization with VRE (Donskey et al., 2000; Taur et al., 2012), suggesting that commensal anaerobes are critical for suppression of VRE and likely Enterococci in general. Commensal bacterial inhibition of VRE is mediated, in part, by stimulation of innate immune defenses (e.g. release of Toll-like receptor ligands) that promote intestinal epithelial cell expression of RegIIIγ, an antimicrobial C-type lectin that inhibits VRE growth in the small intestine (Brandl et al., 2008) (Fig 2A). In a randomized trial of children with VRE infection, oral administration of *Lactobacillus rhamnosus* GG reduced intestinal colonization with VRE (Szachta

et al., 2011), potentially by competing with VRE at the level of binding to intestinal mucus, given that the pili of these two bacterial species share sequence similarities (Tytgat et al., 2016). Fecal transplantation can clear VRE from the mouse intestine and correlates with the presence of Barnesiella (Ubeda et al., 2013) in the colon. Direct inhibition of VRE is mediated by obligate anaerobes, including *Blautia producta* and *Clostridium bolteae* (Caballero et al., 2017), by mechanisms that remain to be defined. The endogenous commensal *Enterococcus faecalis* can also directly inhibit competing Enterococcus strains by expressing bacteriocins (Kommineni et al., 2015).

1.8 Clostridium difficile

The most common hospital-acquired pathogen is *C. difficile*, and infection is generally associated with previous antibiotic administration (Abt et al., 2016). *C. difficile* can cause severe colitis and often occurs in patients with a compromised microbiota. The global rise of two epidemic *C. difficile* strains was recently correlated with their distinct ability to metabolize the disaccharide trehalose, which was introduced as a food additive just prior to the emergence of the antibiotic-resistant strains (Collins et al., 2018).

Several recent studies have identified mechanisms by which the intact intestinal microbiota confers resistance to *C. difficile* colitis. Spores of *C. difficile*, which can survive for long periods of time on dry surfaces, express a receptor that responds to primary bile salts such as taurocholate in the mammalian gastrointestinal tract, inducing germination (Francis et al., 2013). Certain commensal bacteria, such as *Clostridium scindens*, combat *C. difficile*

colonization in part by converting primary bile salts to secondary bile salts, leading to the production of deoxycholic acid and lithocholic acid, which inhibit vegetative growth of *C. difficile* (Buffie et al., 2015; Wilson, 1983) (Fig 2B). Another inhibitory mechanism involves microbiota-mediated depletion of monosaccharides, such as sialic acid, that promote *C. difficile* growth. Antibiotic treatment can eliminate commensals that metabolize sialic acid, thereby increasing sialic acid concentrations in the colon to the benefit of *C. difficile* (Ng et al., 2013). Commensal organisms also cleave sialic acids from host glycoproteins, and thus *C. difficile* growth depends on antibiotic-mediated elimination of bacteria that catabolize sialic acid while preserving bacteria that liberate sialic acid from mucosal glycoconjugates. Antibiotic treatment also leads to transient increases in the luminal concentration of succinate, which can also boost growth of *C. difficile* in the lower gastrointestinal tract (Ferreyra et al., 2014). SCFAs have also been implicated in resistance to *C. difficile* infection (Rolfe, 1984), with dietary fiber and consequent production of the SCFAs acetate, propionate and butyrate enhancing *C. difficile* clearance from the mouse gut (Hryckowian et al., 2018).

The high rate of recurrence following antibiotic treatment of *C. difficile* infection likely results from persistent damage to the microbiota, regardless of which antibiotic regimen is used (Cornely et al., 2014). A randomized clinical trial demonstrated that fecal microbiota transplantation (FMT) is highly effective at curing recurrent *C. difficile* infection (van Nood et al., 2013). A key factor in preventing recurrence and achieving remission is restoring a ‘healthy’ microbiota,

specifically Bacteroides, Lachnospiracaeae, and Ruminoccaceae species (Schubert et al., 2014); (Schubert et al., 2015). Provision of strains of Lachnospiraceae, Lactobacillus, Bifidobacterium, and Lactococcus have also shown varying degrees of success in preventing *C. difficile* recurrence in vitro and in mouse models, but further work needs to be done to optimize which consortia of strains are optimal for prevention (Reeves et al., 2012); (Schoster et al., 2013); (Le Lay et al., 2016). Recent analyses of donor and recipient microbiota, in the setting of FMT in patients with recurrent *C. difficile* infection, led to a model whereby the abundance and phylogeny of the donor and (pre-transplant) recipient microbiota could be used to predict successful microbial engraftment and might ultimately facilitate the assembly of an specific bacterial consortia that optimizes engraftment (Smillie et al., 2018).

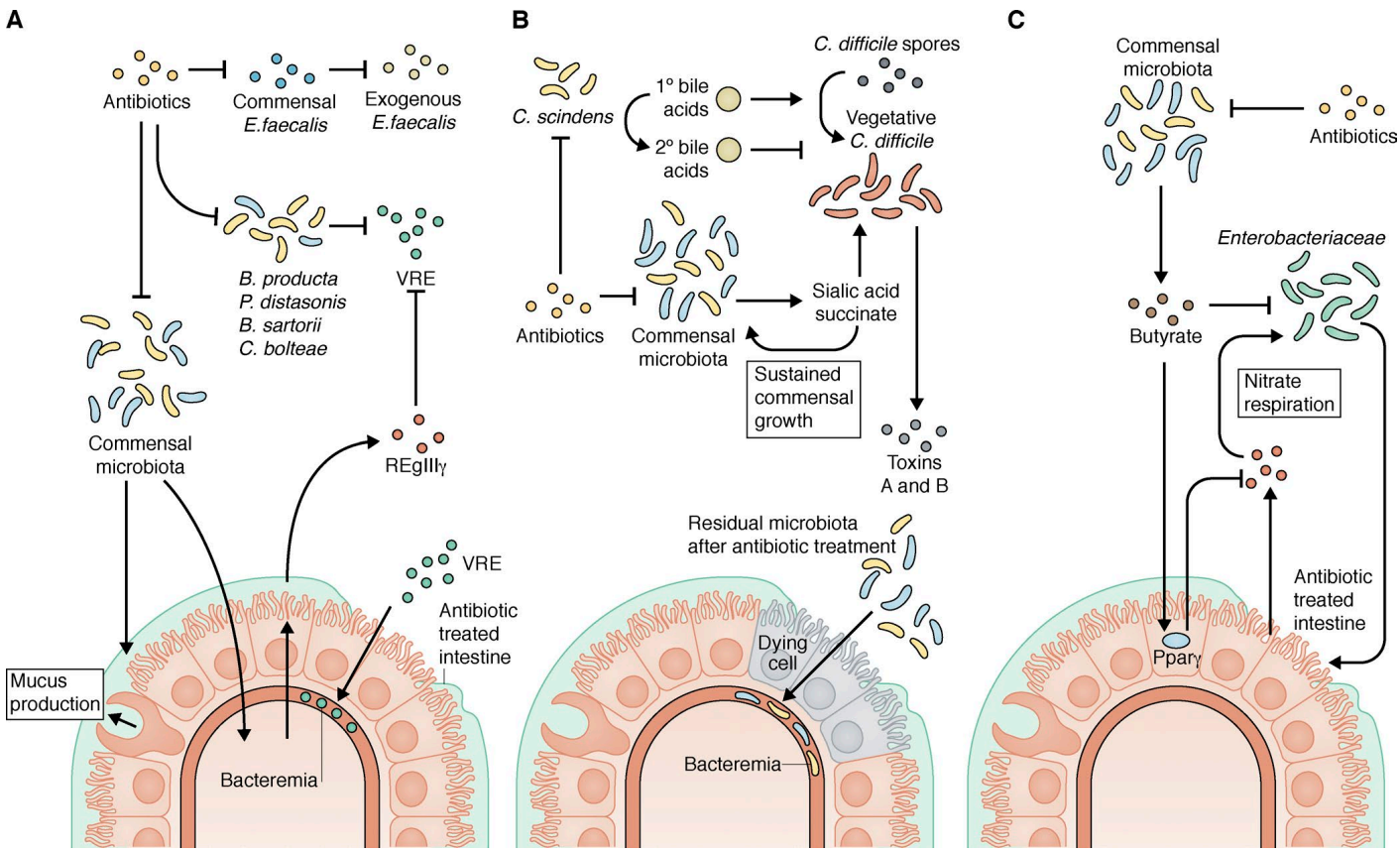

Figure 2: The microbiota drives defense against nosocomial bacterial pathogens

(A) Enterococcal infections can be deleterious to the antibiotic treated host, as Enterococci can translocate into the bloodstream. Loss of colonization resistance is an important component in the manifestation of these infections, and colonization resistance is mediated through several mechanisms, including direct inhibition by commensal strains of *E. faecalis* and obligate anaerobes such as *Blautia producta*, *Parabacteroides distasonis*, *Bacteroides sartorii*, and *Clostridium bolteae*. Bacterial derived TLR ligands drive indirect inhibition by stimulating production of Regenerating islet-derived protein IIIγ (RegIIIγ). (B) *Clostridium difficile* infection can cause severe colitis by inducing epithelial cell death, and this loss of epithelial integrity allows for residual bacteria remaining after antibiotic treatment to spill into the underlying tissue and bloodstream. Spores of *C. difficile* are ingested by the host and germinate into vegetative cells upon stimulation by primary bile acids. When the microbiota is unperturbed by antibiotics, bacteria such as *Clostridium scindens* are present and can convert primary bile acids into secondary bile acids which inhibit vegetative cell growth. Succinate is a metabolic byproduct of commensal bacteria and sialic acid is a

host-derived carbohydrate that is cleaved from epithelial cells by commensals and released into the intestinal lumen. At steady state succinate and sialic acid support sustained growth of various commensal species, but when antibiotics are administered, the commensal species that would benefit from these factors are eliminated, leaving them to be utilized by vegetative *C. difficile* to facilitate its own growth instead. (C) Enterobacteriaceae are a family of bacteria that are adept at exploiting the antibiotic treated intestine by inducing inflammation, a setting in which Enterobacteriaceae can exploit to facilitate their own expansion. Commensal bacteria produce butyrate as a byproduct of carbohydrate fermentation, which in turn prevents inflammation and also directly kills Enterobacteriaceae in the presence of acidified pH. Loss of butyrate production reduces PPARγ signaling in epithelial cells, inducing iNOS expression that can be used as a substrate for nitrogen respiration in Enterobacteriaceae. This increased availability of iNOS is exploited by Enterobacteriaceae, creating a positive feedback loop that enables expansion of these opportunistic pathogens since increased presence of Enterobacteriaceae can in turn lead to increased expression of iNOS.

1.9 Enterobacteriaceae

Infections caused by Gram-negative rods belonging to the Enterobacteriaceae family of the Proteobacterium phylum are particularly problematic in healthcare settings. This family includes pathogenic organism such as *Salmonella enteritidis*, *Shigella flexneri* and *Yersinia enterocolitica*, but also many other members that are less virulent and are common residents of the mammalian intestinal tract, including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes* and *Enterobacter cloacae*. As facultative anaerobes, these bacteria inhabit the length of the gastrointestinal tract, from the oral cavity to the anaerobic colon. The density of Enterobacteriaceae colonization is generally low, rarely contributing more than a fraction of 1% to the colonic microbiota. However, following antibiotic treatment, these organisms can undergo marked expansion and can achieve over 90% occupancy of the lower gastrointestinal tract in some settings (Taur et al., 2012). This scenario has become increasingly common in clinical settings as organisms like *E. coli* and *K.*

pneumoniae have acquired resistance against a wide range of antibiotics, in some circumstances all those that are clinically available.

The mechanisms by which antibiotic-naïve microbiota confers colonization resistance against Enterobacteriaceae are manifold but can be divided into three main groups: direct microbe-to-microbe inhibition; competition for nutrients such as carbohydrates, iron, zinc and manganese; and indirect inhibition via activation of the host immune system or modification of host factors. Because the Enterobacteriaceae family includes important gastrointestinal pathogens, much work on microbiota-mediated colonization resistance has focused on pathogens (Salmonella and Yersinia in particular), but findings from these studies likely apply to Enterobacteriaceae in general.

Of particular recent interest has been the finding that Enterobacteriaceae undergo expansion during inflammation of the gut (Lupp et al., 2007). Deeper studies of this phenomenon revealed that *S. typhimurium*, for example, exploits inflammation and associated reactive oxygen species by using tetrathionate as a respiratory electron acceptor (Winter et al., 2010). More recent studies have demonstrated that antibiotic-induced loss of butyrate reduces PPARg signaling and thereby induces gut inflammation and iNOS expression, providing *E. coli* with a growth advantage because it can use nitrates as a respiratory electron acceptor (Byndloss et al., 2017) (Fig 2C). The host inflammatory response includes production of calprotectin, a molecules that sequesters zinc and manganese, thereby depriving pathogenic microbes of essential nutrients. But *S. enterica* combats this by encoding metal transporters that outcompete host-

mediated chelation of manganese (Diaz-Ochoa et al., 2016; Liu et al., 2012). In the setting of intestinal inflammation, competition between members of the Enterobacteriaceae family can be mediated by small, bacterial proteins called microcins that enable certain strains of *E. coli*, for example, to expand in the intestinal lumen (Sassone-Corsi et al., 2016). This form of colonization resistance has recently been exploited by engineering an *E. coli* strain that encodes a tetrathionine-inducible microcin, resulting in resistance to Salmonella infection (Palmer et al., 2018).

One of the most important mechanisms of growth restriction of Enterobacteriaceae is mediated by short-chain fatty acids (SCFAs) such as acetate and butyrate, particularly at low pH. Early studies showed that expansion of Salmonella in the mouse colon was inhibited by acetate at low pH, but not high pH, and that antibiotic treatment increased the luminal pH (Bohnhoff et al., 1964b). The widespread use of *E. coli* for the production of recombinant proteins led to the discovery that acetate and butyrate are protonated at low pH, allowing them to diffuse across the bacterial membrane and subsequently acidify the bacterial cytoplasm, inhibiting bacterial growth (Booth, 1985). This general process of fermentative acidification has been used for centuries to preserve food by inhibiting the growth of pathogens during storage (Levine and Fellers, 1940). Acetate production by Bifidobacteria protects mice against enteropathogenic *E. coli* infection, with inhibition attributed to acetate-mediated enhancement of mucosal epithelial resistance to secreted bacterial enterotoxins (Fukuda et al., 2011).

The identity of commensal bacterial species that inhibit Enterobacteriaceae in the lower gastrointestinal tract has been investigated most extensively with *Salmonella enterica*. The importance of obligate anaerobes in inhibition of *S. enterica* was recognized by Bohnhoff over 50 years ago (Bohnhoff et al., 1964a), and more recent studies have correlated the presence of specific commensal species with enhanced resistance to *S. enterica* infection (Brugiroux et al., 2016) (Sassone-Corsi et al., 2016). FMT has been demonstrated to clear dense intestinal colonization with *Klebsiella pneumoniae* in mice (Caballero et al., 2015), and some examples of FMT-mediated clearance of antibiotic-resistant bacteria suggest that this may extend to humans (Bilinski et al., 2016); (Crum-Cianflone et al., 2015). Further studies, however, are necessary to identify the mechanisms by which specific commensal bacteria inhibit the expansion of Enterobacteriaceae in the intestinal lumen.

1.10 Current status of microbiota-mediated inhibition of intestinal pathogens

Over the past decade, the growing focus on the microbiota has greatly increased our understanding of colonization resistance, in part by revealing that the infectiousness of intestinal pathogens can be reduced by multiple, parallel mechanisms. In some cases, recent studies using new experimental platforms and technologies have confirmed old ideas and findings. But novel mechanisms are also being discovered. Undoubtedly we are far from completely understanding microbiota-mediated defenses, in part because there are mechanisms that await discovery but also because the relative contributions of known mechanisms have, so far, been inadequately quantified. Not surprisingly,

the most recently discovered mechanisms tend to gain center-stage attention for a while, only to be replaced by the next discovery which, though more recent, may be quantitatively less impactful. An ongoing challenge, therefore, is to temporally, quantitatively and biogeographically (e.g. inhibition in ileum versus colon) stitch together the various inhibitory mechanisms.

Although the enormous impact of antibiotic treatment on human health and longevity is difficult to overstate, recognition that antibiotics can have adverse effects on health and paradoxically result in increased susceptibility to infection is increasing (Pamer, 2016). While serious bacterial infections require antibiotic administration, remediating post-treatment damage to a patient's microbiota represents a logical, if challenging, subsequent step. With this concept in mind, a recent study demonstrated the feasibility of collecting, characterizing and storing the fecal microbiota of patients prior to hematopoietic stem cell transplantation (which is often associated with marked antibiotic-mediated destruction of the intestinal microbiota), and then successfully re-implanting the patient's own microbiota following stem cell transplant (Taur et al., 2018). For patients undergoing complex medical procedures associated with microbiota loss, reconstitution of the microbiota with the patient's own commensal microbes represents an approach that may reduce the incidence of subsequent infections.

As previously highlighted, the intestinal microbiota is an ecosystem (Costello et al., 2012). Members of ecosystems establish relationships that range from symbiotic to commensal to competitive. Characteristics of the occupied

space, such as temperature, moisture, pH and osmolarity can have enormous impacts on which species flourish, struggle or become extinct. Ecosystem inhabitants modify the spaces they occupy to varying extents. In some circumstances the very existence of the physical space depends on its inhabitants, as is the case with the microbial ecosystem contained within the intestine of humans and other mammals. Thus, competitive interactions between intestinal inhabitants are likely tempered by the need to maintain the health of their host. In the gut, optimal support of the host requires an array of bacterial species that serve digestive, metabolic, developmental and immune-activating functions. From the perspective of a commensal bacterial species that lives in the gut lumen, vanquishing a competing species and conquering its niche may seem like a predominating evolutionary strategy, but the associated loss of microbial diversity would reduce the health of the host and thus damage or even eliminate the environment. Deeper and more complete understanding of the complex relationships between commensal bacterial species, mammalian hosts and invasive pathogens is likely to lead to clinically important approaches to improve human health and resistance to infection.

CHAPTER 2

IMPACT OF ANTIBIOTIC-RESISTANT INTESTINAL BACTERIA ON MUCOSAL IMMUNE ACTIVATION AND ANTIMICROBIAL DEFENSES[2]

2.1 Abstract

Antibiotic treatment of patients undergoing complex medical treatments can deplete commensal bacterial strains from the intestinal microbiota, thereby reducing colonization resistance against a wide range of antibiotic-resistant pathogens. Loss of colonization resistance can lead to marked expansion of Vancomycin-resistant *Enterococcus faecium* (VRE), *Klebsiella pneumoniae* (Kp) and *Escherichia coli* (Ec) in the intestinal lumen, predisposing patients to bloodstream invasion and sepsis. The impact of intestinal domination by these antibiotic-resistant pathogens on mucosal immune defenses and epithelial and mucin-mediated barrier integrity is unclear. We used a mouse model to study the impact intestinal domination by antibiotic-resistant bacterial species and strains on the colonic mucosa. While VRE, Kp, Ec, *Proteus mirabilis* (Pm) and *Enterobacter cloacae* colonized the gastrointestinal tract of antibiotic-treated mice at similar densities and did not induce histologic evidence of epithelial damage, Kp, Pm and *E. cloacae* promoted greater recruitment of neutrophils to the colonic mucosa. Intraluminal localization of bacterial strains varied, with some congregating near the mucin layer and others evenly distributed throughout

[2] Chapter 2 summarizes the work currently in submission Keith, J.W., Becattini, S., Sia, J.K. Gjonbalaj, M., Seok, R., Leiner, I., Littmann, E., and Pamer, E.G. 2019. Impact of antibiotic-resistant intestinal bacteria on mucosal immune activation and antimicrobial defenses.

the colonic lumen. Thickness of the dense mucin layer was influenced by bacterial strains colonizing the colon and their proximity to the epithelium. To test the hypothesis that the residual microbiota can influence the severity of colitis caused by infection with *Clostridioides difficile*, we infected mice that were densely colonized with Kp, Pm, Ec or *E. cloacae* with a virulent strain of *C. difficile* and monitored mortality, weight loss and recovery from infection. Despite the compositional differences in the gut microbiota, the severity of *C. difficile* infection and mortality did not vary significantly between mice colonized with different antibiotic-resistant bacterial species. Our results suggest that the virulence mechanisms enabling *C. difficile* infection (CDI) and epithelial destruction overwhelm the relatively minor impact of less virulent, antibiotic-resistant pathogens on the outcome of CDI.

2.2 Introduction

The human colonic microbiota consists of diverse, predominantly anaerobic bacterial strains belonging to the Bacteroidetes and Firmicutes phyla and represents an ecosystem within the host that is defined by multiple layers of cooperation and competition between its component microbes. The microbiota and its products impact host metabolism and immune development and protect against infection by activating a range of direct and indirect antimicrobial mechanisms that function in parallel and are collectively referred to as colonization resistance. Marked variation in baseline microbiota composition and colonization resistance between individuals likely accounts, at least in part, for the wide range of disease severities associated with infection by specific

microbial pathogens. Although many factors influence microbiota composition and diversity, including diet, environmental exposure and host immune status, wide spread use of antibiotics has had a particularly large impact on the microbiota of human and domesticated animal populations. While antibiotics dramatically reduce mortality resulting from bacterial infections, antibiotic-induced collateral damage to the microbiota impairs colonization resistance and, paradoxically, increases susceptibility to infection.

Commensal microbes inhabiting the lower intestinal tract are generally antibiotic sensitive and inhibited by administration of broad-spectrum antibiotics. Loss of commensal bacteria is particularly common in patients undergoing cancer treatment and is associated with the expansion of antibiotic-resistant pathobionts, such as Vancomycin-resistant *Enterococcus faecium* (VRE), *Klebsiella pneumoniae* (Kp) and *Escherichia coli* (Ec). In patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT), the incidence of intestinal domination by VRE exceeds 50%, while domination by Kp or Ec occurs in up to 20% of patients (Taur et al., 2012). Intestinal domination in allo-HSCT patients is associated with an increased incidence of bloodstream infection by the respective colon-dominating pathogen.

Commensal bacteria belonging to the Bacteroidetes and Firmicutes phyla impact immune development at the levels of T cell development and differentiation and Kp colonization of the gut has been associated with differentiation of Th1 CD4 T cells in the colon (Atarashi et al., 2017; Furusawa et al., 2013). While commensal-mediated immune differentiation requires consortia

of bacterial species (Atarashi et al., 2013; Atarashi et al., 2011; Furusawa et al., 2013), colonization of germ-free mice with individual commensal species can also impact T cell development (Ivanov et al., 2009) (Mazmanian et al., 2008). It remains unclear, however, whether antibiotic-resistant pathobionts that commonly dominate the gut and cause systemic infections in hospitalized patients impact mucosal immune defenses, intestinal epithelial integrity and mucus production. Furthermore, it is unclear whether intestinal domination by common, antibiotic-resistant bacterial species impacts patient susceptibility to one of the leading causes of nosocomial infection, *Clostridiodes difficile*.

To address these issues, we used a mouse model to investigate intestinal domination by a range of antibiotic-resistant bacterial strains isolated from patients undergoing cancer treatment. We found marked differences in the localization of different bacterial species and strains in the colons of mice. Proximity of different antibiotic-resistant strains to the epithelial layer and the thickness of the mucus layer varied between strains and resulted in differences in the induction of inflammatory and anti-inflammatory cytokines in the intestinal mucosa. Despite differences in the state and intensity of immune activation, we did not detect a significant impact of intestinal domination by different antibiotic-resistant bacterial strains on the course and severity of infection by *C. difficile*. These findings indicate that the pathogenetic mechanisms engaged by *C. difficile* to cause colitis, and the host's immune defenses leading to resolution, override the relatively minor impact of dominating bacterial species on colonic immune defenses.

2.3 Antibiotic treated mouse microbiota is receptive to ampicillin-resistant bacterial species

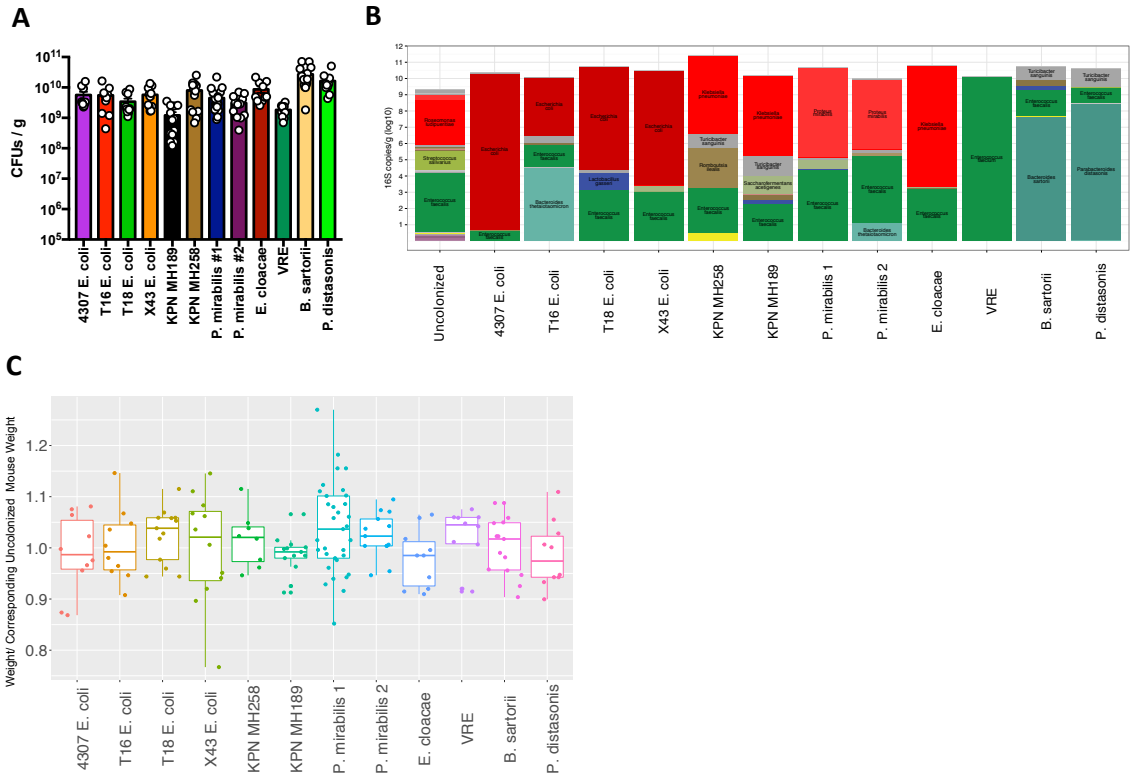

Figure 3: Antibiotic treated mouse microbiota is receptive to ampicillin-resistant bacterial species.

(A) Wildtype C57BL/6 mice (n ≥ 10 per group, results from 17 independent experiments) were treated with ampicillin in drinking water (0.5g/L) for four days and then inoculated with $2x10^4$ CFUs of one of twelve ampicillin-resistant bacterial strains. Two days after inoculation mice ampicillin was removed from drinking water and fecal pellets were one day later and CFUs were quantified. **(B)** DNA was extracted from fecal pellets and subjected to 16S rRNA PCR amplification and sequencing of the V4-V5 region. The average abundance of bacterial taxa for all mice of the same group is plotted. **(C)** The weight of mice in each group was divided by the weight of control mice to assess weight loss associated with colonization by ampicillin-resistant bacterial strains.

2.4 Intestinal domination by ampicillin-resistant bacterial strains

Bacterial strains isolated from hospitalized patients, particularly those belonging to the Enterobacteriaceae family, are frequently resistant to ampicillin.

Ampicillin-resistant strains of *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis* and bacterial species belonging to the Bacteroidetes phylum, particularly following antibiotic treatment, can reach high densities in the intestines of colonized patients. The impact of intestinal domination, herein defined as exceeding 30% frequency by 16S rRNA sequence analysis, upon the host's mucosal immune and antimicrobial defenses is largely undefined. To begin to address this knowledge gap, we obtained 10 ampicillin-resistant clinical isolates of *K. pneumoniae*, *E. coli, Enterobacter cloacae, P. mirabilis* and vancomycin resistant *Enterococcus faecium* (Xiong et al., 2015) (Sorbara et al., 2019) from patients undergoing cancer treatment and ampicillin-resistant strains of *Bacteroides sartorii* and *Parabacteroides distasonis* from a mouse colony that had been treated for over 10 years with ampicillin (Caballero et al., 2017). To study the impact of these ampicillin-resistant strains, we depleted the indigenous microbiota of wildtype C57BL/6 mice with ampicillin for four days and inoculated mice by oral gavage with $2x10^4$ CFUs of each strain. On day 2 post inoculation, we discontinued ampicillin treatment and obtained fecal pellets one day later to quantify CFUs by selective plating (Fig. 3A). Although there were some differences in the densities of fecal colonization by the different ampicillin resistant bacterial strains, all achieved densities exceeding 10^9 per gram of feces.

To determine the microbiota composition of mice colonized with ampicillin-resistant bacterial strains, we performed 16S rRNA sequencing. Mice receiving ampicillin followed by inoculation with an ampicillin-resistant bacterial strain

developed domination, as demonstrated in Fig. 3B. Of note, because ampicillin treatment had been discontinued for 24 hours prior to fecal pellet collection, endogenous bacterial strains were detected in all mice. We also detected bacterial populations, albeit at lower density as determined by quantitative 16S rRNA gene PCR (Fig. 3B, left column), in mice that were treated with ampicillin but not exposed to ampicillin-resistant bacterial strains. These strains likely represent residual bacteria that survived ampicillin treatment and underwent re-expansion following discontinuation of ampicillin treatment. With the exception of those exposed to VRE, ampicillin-treated mice had blooms of *Enterococcus faecalis*, suggesting that *E. faecium* provides colonization resistance against *E. faecalis,* possibly by bacteriocin production (Kommineni et al., 2015). Depending on the circumstances, *E. faecalis* has been implicated as a pathogen or a harmless commensal. Since mice did not lose weight or demonstrate evidence of intestinal pathology, we do not believe that the presence of *E. faecalis* impacted the outcome or our study (Fig. 3C). Collectively, these data show that we can substantially alter the microbiota of ampicillin treated mice by dominating their intestinal bacterial composition with a range of ampicillin-resistant bacterial isolates.

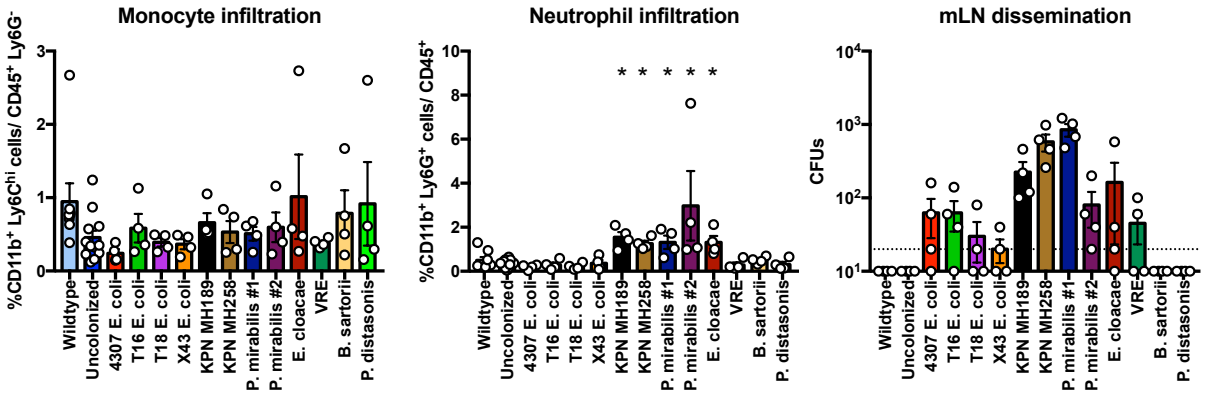

Figure 4: Antibiotic-resistant bacterial strains exhibit distinct capacity to disseminate to mLNs and recruit neutrophils to the colonic lamina propria

Wildtype C57BL/6 mice (n=4-5 per group) were treated with metronidazole, vancomycin, and neomycin followed by ampicillin and then inoculated with $2x10^4$ CFUs of the antibiotic-resistant bacterial strains. Five days following inoculation, cells isolated from the colonic lamina propria were profiled by flow cytometry. Bacterial dissemination to mLNs was determined by selective plating of whole mLN.

2.5 Ampicillin-resistant bacterial strains localize and activate the mucosal immune system distinctly

Although each member of our panel of ampicillin-resistant bacterial strains colonized mice treated with ampicillin, and none of the colonized mice became overtly ill, it was unclear whether the different bacterial strains impacted the mucosal immune system or localized distinctly within the gut. To address these questions, we treated wildtype C57BL/6 mice with metronidazole, neomycin, and vancomycin for 3 days followed by ampicillin for four days and then administered $2x10^4$ CFUs of each of the twelve ampicillin-resistant strains by oral gavage. Colonic monocyte and neutrophil infiltration and cytokine expression and bacterial dissemination to mesenteric nodes, spleen, liver and blood were determined.

Flow cytometric analyses demonstrated that monocyte infiltration of the

colonic lamina propria was not influenced by intestinal colonization with different ampicillin-resistant bacterial species (Fig. 4). In contrast, neutrophils were more abundant in the colonic lamina propria of mice densely colonized with *K. pneumoniae, P. mirabilis* or *E. cloacae* and these bacterial strains had the highest levels of dissemination to mesenteric lymph nodes (mLN). Although *B. sartorii* and *P. distasonis* densely colonized the colon, these obligate anaerobes were not detected by culture in mLNs.

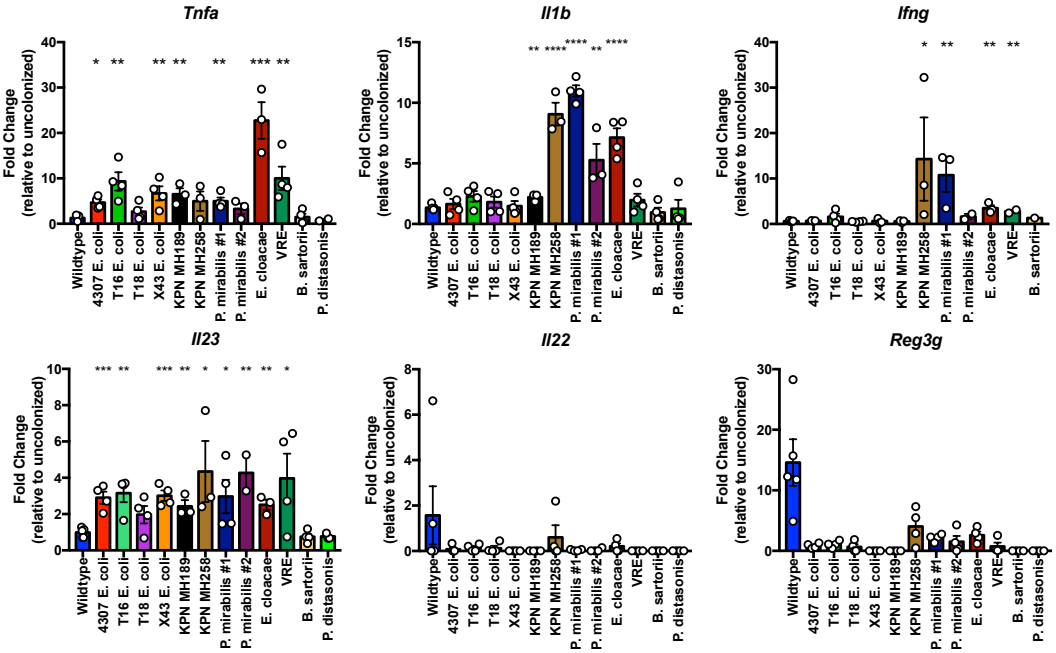

Figure 5: Impact of antibiotic-resistant bacterial strains on transcription of cytokines in the colon

Wildtype C57BL/6 mice (n=4-5 per group) were treated with metronidazole, vancomycin, and neomycin followed by ampicillin and then inoculated with $2x10^4$ CFUs of the antibiotic-resistant bacterial strains. Five days following inoculation, RT-PCR was performed on whole colonic tissue. Fold induction of the indicated cytokines relative to antibiotic treated, but uncolonized mice is plotted. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data shown are ± SEM.

To determine whether members of the ampicillin-resistant bacterial panel triggered transcription of genes encoding inflammatory cytokines, we performed RT-PCR for *Tnfα, Il1β, Ifnγ, Il23, Il22* and *Reg3γ* (Fig. 5). These cytokines have been implicated in mucosal defense against intestinal pathogens, including *Clostridium difficile* (Abt et al., 2015); (Cowardin et al., 2015); (Hasegawa et al., 2012); (Hasegawa et al., 2014). *Tnfα* and *Il23* transcription was significantly increased in most mice that had been colonized with an ampicillin-resistant bacterial strain. *Il1β* expression generally correlated with increased neutrophil infiltration, with the exception of colonization by *K. pneumoniae* MH189, which was associated with increased neutrophil levels but baseline *Il1β* expression. Mice colonized with *K. pneumoniae* MH258, *P. mirabilis #1*, VRE and *E. cloacae* had increased *ifn*γ transcription.

*Tnf*α*, Il1β*, and *Ifnγ* transcription was induced by some ampicillin resistant bacterial strains, however we did not detect induction of *Il22* and *Reg3γ* transcription. While *B. sartorii* and *P. distasonis* did not induce *Il23* transcription, the other ampicillin-resistant strains induced 2- to 4-fold increased *Il23* transcription, but this did not lead to increased transcription of either *Il22* or *Reg3γ*. Previous studies have demonstrated that MyD88-mediated induction of IL23 drives expression of IL22 by innate lymphocytes in the lamina propria, which induces Reg3γ expression in intestinal epithelial cells (Brandl et al., 2007); (Vaishnava et al., 2008; Vaishnava et al., 2011). Our results suggest that the low level of IL23 transcript induction by the ampicillin-resistant strains is insufficient to promote IL22 and Reg3γ expression.

Since it is likely that microbial molecules released by colonizing bacteria stimulate innate immune receptors in the gut wall, we hypothesized that spatial localization of ampicillin-resistant bacterial strains, particularly proximity to intestinal epithelial cells, might determine the degree of mucosal immune activation. To address this, we densely colonized mice with each of the ampicillin-resistant isolates and performed Fluorescent In-situ Hybridization (FISH) to localize bacteria within the colonic lumen while counterstaining for mucus and nuclei. In wild type mice (Fig. 6A), we detected dense bacterial populations that increased in density near the epithelium, with the greatest density abutting the dense mucus layer. In contrast, mice that had been treated with antibiotics had a very thin, patchy mucus layer and a paucity of bacteria detected by FISH. The luminal localization varied for different ampicillin-resistant bacterial strains (Fig. 6B), with some strains, such as X43 *E.coli, K. pneumoniae* MH258, *E. cloacae,* VRE, *B. sartorii,* and *P. distasonis* congregating near the mucus layer while other strains were localized throughout the lumen (Fig. 7).

We also observed differences in mucus thickness and continuity and the extent to which bacteria were residing within mucus in mice reconstituted with different bacterial strains. Plotting the proportion of bacteria at different points along the radius extending from the center of the intestinal lumen to the intestinal epithelium (Fig. 8A) demonstrates that while ampicillin-resistant strains are present throughout the luminal space, some strains localize most prevalently in close proximity to the epithelium (>8% within 100 μm of the epithelial surface).

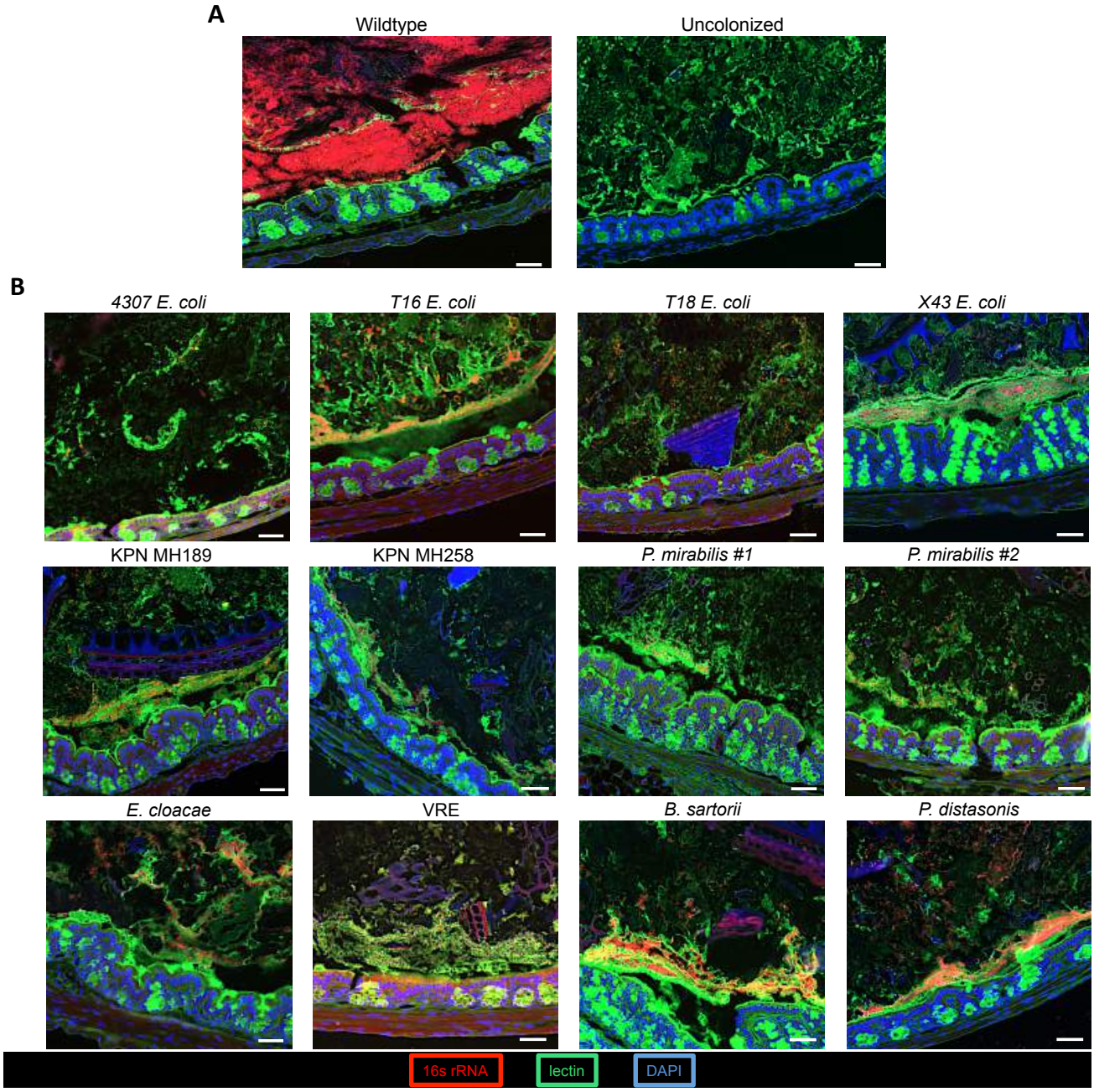

Figure 6:Antibiotic-resistant bacterial strains preferentially reside in proximity to the mucus layer

(A & B) Colonic sections were subjected to fluorescent in-situ hybridization (FISH). Entire colon cross-sections from control and antibiotic treated mice, and mice densely colonized with each of the twelve antibiotic-resistant strains were stained with a pan-bacterial FISH probe targeting 16S rRNA. Sections were counterstained with fluorescein-conjugated lectins to visualize intestinal mucus and Hoechst dye to visualize host nuclei. Images are representative of at least 4 mice per group. Scale bar = 200μm.

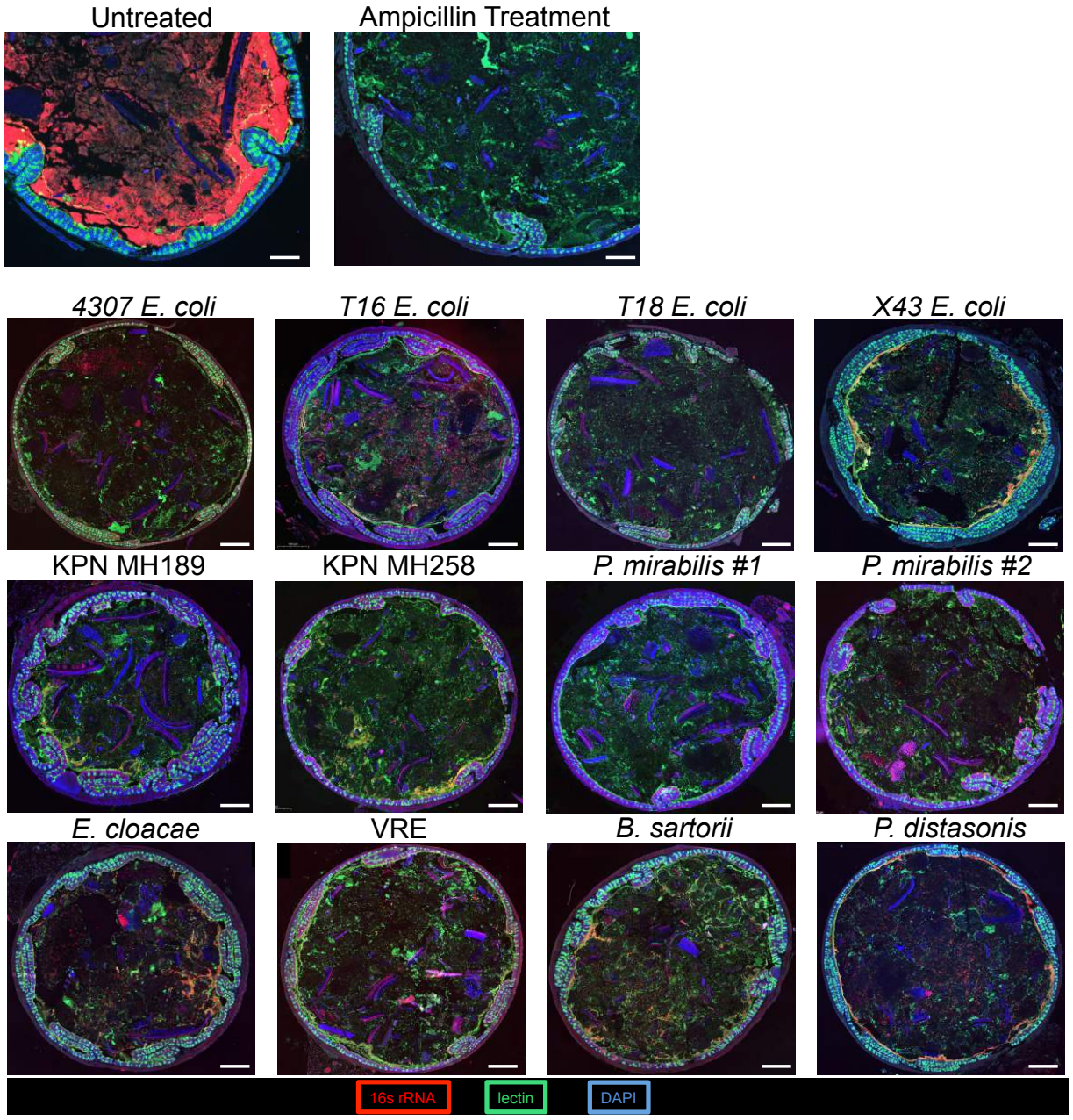

Figure 7: Antibiotic resistant strains localize distinctly and impact the host colonic mucus layer

Colonic sections were subjected to fluorescent in-situ hybridization (FISH). Entire colon cross-sections from control and antibiotic treated mice, and mice colonized with each of the twelve antibiotic-resistant strains were stained with a pan-bacterial FISH probe targeting the 16S rRNA. Sections were counterstained with fluorescein-conjugated lectins to visualize intestinal mucus and Hoechst dye to visualize host nuclei. Images are representative of at least 4 mice per group. Scale bar = 500μm.

Microbiota composition impacts mucus thickness (Fig 8B), and the mucus layer detected in wild type mice is reduced by antibiotic treatment. While most ampicillin-resistant bacterial strains partially restored mucus thickness, two of the tested strains (*E. coli* 4307 and T18*)* did not, potentially a result of their increased distance from the epithelial surface compared to *E. coli* T16 and X43*.* These data demonstrate that despite reaching similar levels of overall bacterial density inside the intestinal lumen, bacterial strains localize distinctly with the lumen, potentially impacting mucus thickness and bacterial dissemination and altering the host's level of mucosal immune activation.

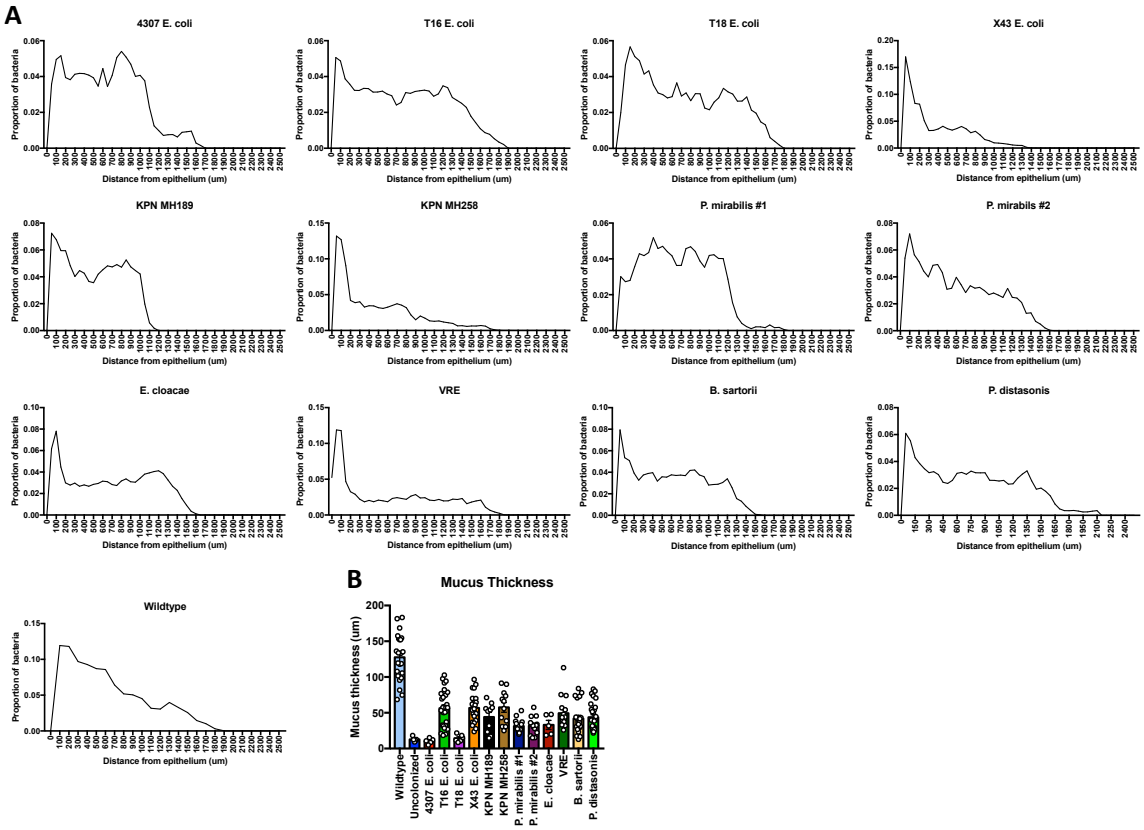

Figure 8: Antibiotic-resistant bacterial strains localize distinctly in the colon lumen and influence mucus thickness

(A) Distance of fluorescent bacteria from the epithelial cell layer was measured

using confocal microscopy. **(B)** Thickness of the detectable mucus layer in mice densely colonized with each of the antibiotic-resistant isolates, compared to wildtype mice that were untreated and mice that were treated with antibiotics but were not colonized post antibiotic treatment.

2.6 Microbiota composition does not significantly influence survival following C. difficile infection

Clostridioides difficile is a common cause of antibiotic-associated diarrhea and is particularly prevalent in patients who have sustained damage to their intestinal microbiota. The interactions between host, indigenous microbiota and pathogens are complex and each component contributes to the pathogenesis of *C. difficile* infection (CDI). The severity of *C. difficile* infections varies, however, with some patients having only mild disease and others developing extensive necrotizing colitis. Host factors, such as age, underlying diseases and immune status and virulence differences between *C. difficile* strains contribute to disease severity but do not completely explain the wide range of disease manifestations. One hypothesis that remains unexplored is whether the residual microbiota composition at the time of a *C. difficile* infection contributes to colitis severity. Host mechanisms underlying recovery from acute CDI remain incompletely defined and might be influenced by microbiota composition. To address this, wild type C57BL/6 mice were colonized with each of the ampicillin-resistant strains and inoculated by oral gavage with approximately 200 spores of the virulent *C. difficile* strain VPI 10463 (Fig. 9A).

As noted earlier, all twelve ampicillin strains achieved high density in the mouse intestine and persisted throughout *C. difficile* infection. None of the strains provided colonization resistance or clearance of *C. difficile* for 7 days

following inoculation (Fig. 9B). The density of *C. difficile* was similar in mice colonized with the different bacterial strains and there were no statistically significant differences in survival following *C. difficile* infection (Fig. 9C)

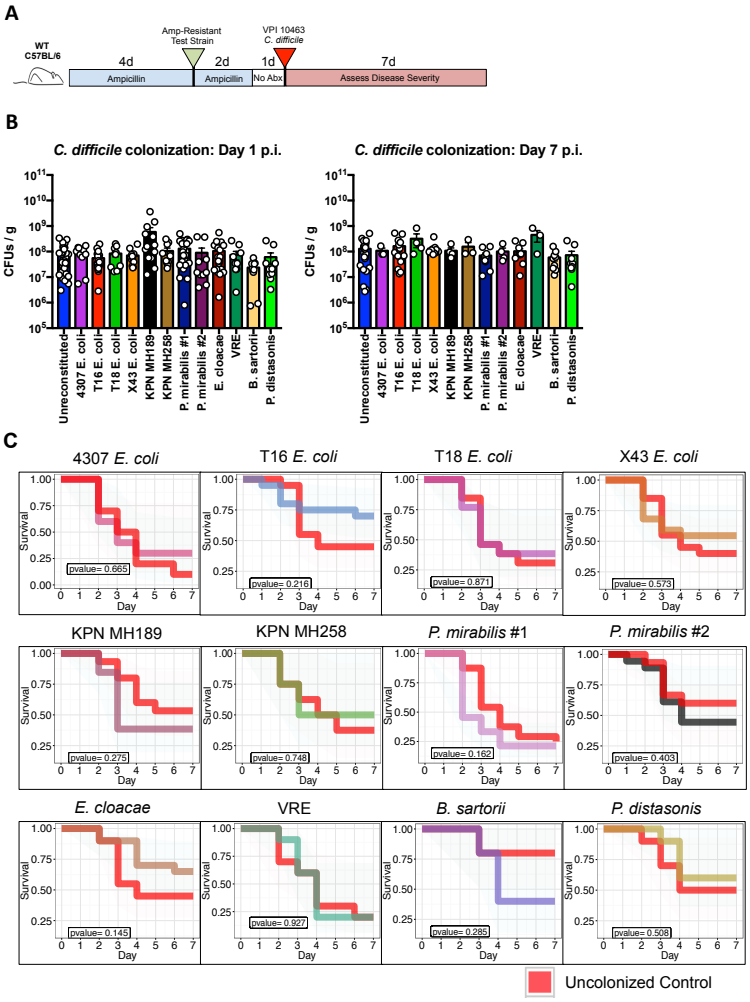

Figure 9: Residual intestinal microbiota composition does not significantly influence survival following C. difficile infection

(A) Scheme of experimental design to determine impact of residual microbiota composition on *C. difficile* infection outcome **(B)** After ampicillin treated wildtype C57BL/6 mice (n ≥ 10 per group, results from 17 independent experiments) were colonized with each of the ampicillin-resistant clinical isolates, mice were infected with 200 spores of *C. difficile* VPI 10463 via oral gavage. On day 1 and day 7 post infection, *C. difficile* CFUs in fecal pellets were quantified using selective plating. **(C)** Survival was monitored for one week post *C. difficile* infection

K. pneumoniae, P. mirabilis, and *E. cloacae* strains were the most immunomodulatory, in terms of inducing neutrophil recruitment to the colon, induction of proinflammatory cytokines, proximity to the epithelium, invasion of the dense mucus layer and dissemination to mLNs. Despite these features, these strains did not impact survival following *C. difficile* infection. *C. difficile* infected control mice general had a 40% survival rate.

2.7 Background microbiota influences host immune response, C. difficile virulence, and bacterial dissemination in the setting of CDI

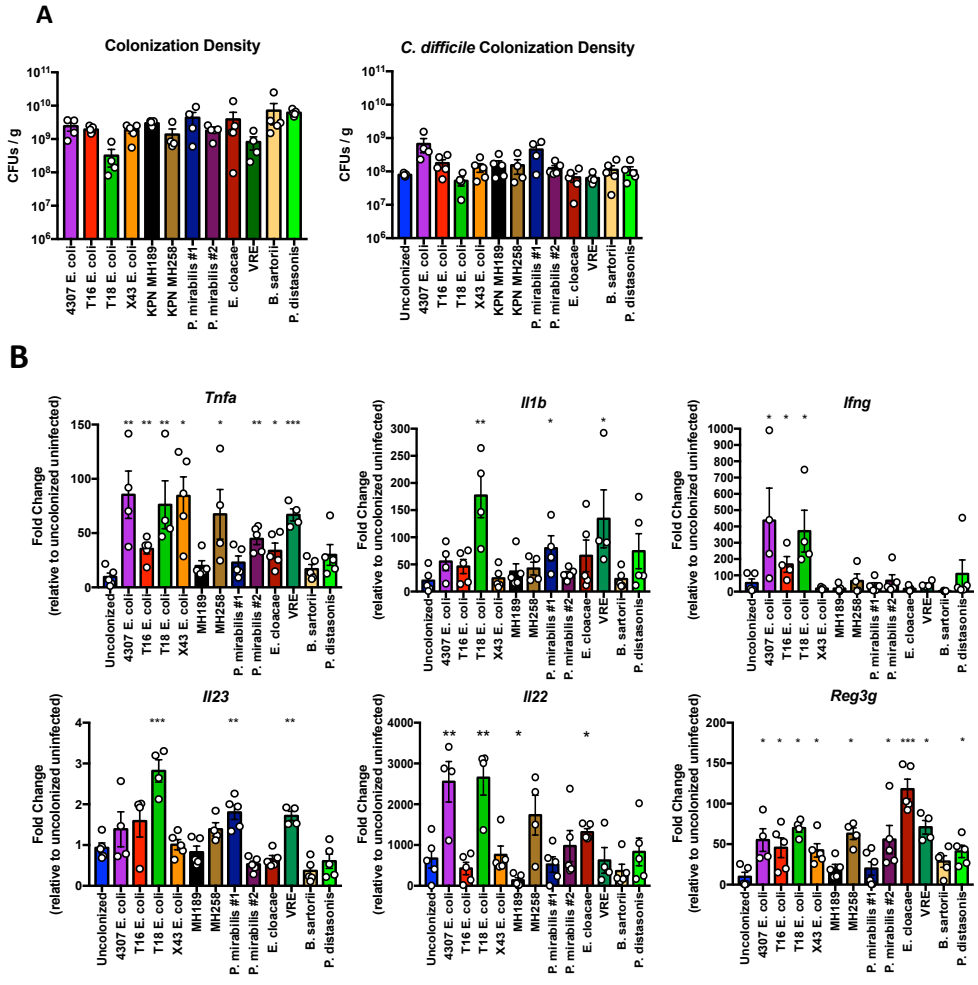

Figure 10: Impact of residual microbiota on host immune responses during CDI

(A) Mice were inoculated with each antibiotic-resistant isolate followed by 200 spores of *C. difficile* VPI 10463 and euthanized 2 days later. CFUs of the antibiotic-resistant strain and *C. difficile* in cecal contents were quantified by selective plating. **(B)** Fold induction of immune cytokines in colonic tissue where determined by quantitative RT-PCR.. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to control mice. Data shown are ± SEM.

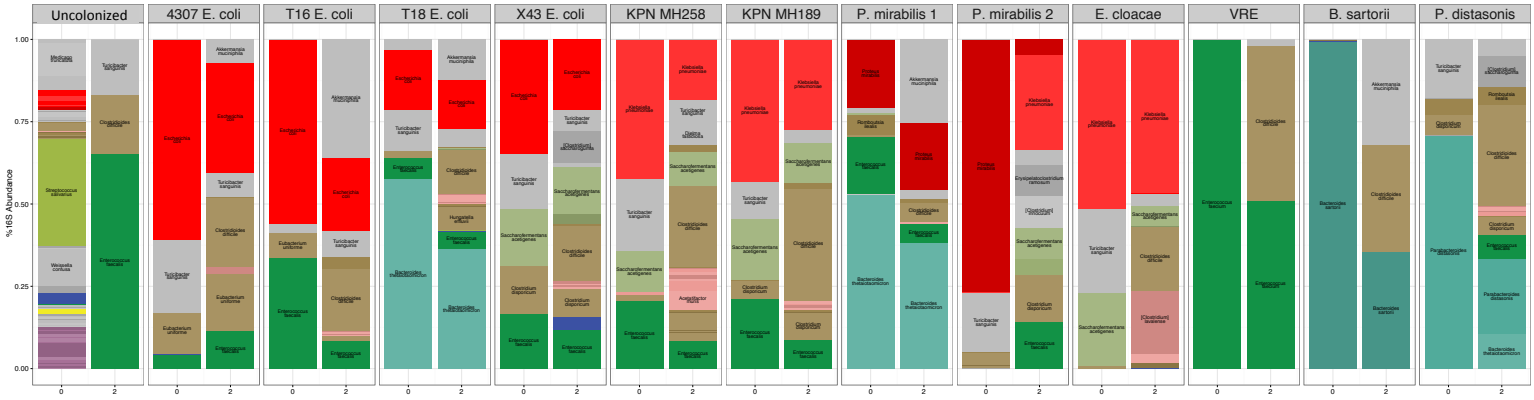

Figure 11: Changes in mouse microbiota composition following *C. difficile* infection

C57BL/6 mice were infected with 200 spores of C. difficile (VPI 10463 strain) following antibiotic pretreatment. DNA was extracted from fecal pellets and subjected to 16S rRNA PCR amplification and sequencing of the V4-V5 region. The average abundance of bacterial taxa for all mice of the same group is plotted both on day 0 and day 2 post infection.

To determine whether the presence of different antibiotic-resistant bacterial strains influence the mucosal immune response to *C. difficile* infection, wildtype C57BL/6 mice were colonized with each ampicillin-resistant bacterial strain and infected with *C. difficile* VPI 10463 and cecal content and tissue were isolated 2 days following infection. All mice had comparable cecal content colonization levels with the ampicillin-resistant isolate they were administered and *C. difficile* (Fig. 10A). *C. difficile* infection reduced the density of the ampicillin-resistant strains in the intestinal contents compared to uninfected mice.

Antibiotic-treated mice had blooms of *Enterococcus faecalis,* with the exception of VRE, *B. sartorii,* and *E. cloacae* dominated mice (Fig. 11). We found that colonization of antibiotic-treated mice with antibiotic-resistant bacterial strains prior to *C. difficile* infection resulted in increased transcription of inflammatory cytokine genes (Fig. 10B). Most bacterial strains induced significant increases in *Tnf*α transcription compared to *C. difficile* infected mice that had not received antibiotic-resistant bacterial strains. T18 *E. coli, P. mirabilis #1* and VRE colonization increased transcription of genes encoding Il1β and Il23, which have been shown to adversely impact CDI outcomes (Buonomo et al., 2013). *Il22* transcription was dramatically increased in the setting of CDI and was associated with increased levels of *Reg3*γ transcription. Mice with the thinnest mucus layers (4307 *E. coli* and T18 *E. coli*) had the highest levels of *Ifn*γ and *Il22* transcripts. On day 2 post *C. difficile* infection, we performed H&E staining on proximal colonic tissue sections (Fig. 12A and Fig. 13). Slides were assessed in a blinded fashion and histologically scored, revealing that *K. pneumoniae* MH189 and VRE had increased tissue pathology, consistent with the finding that their cecal contents had higher *C. difficile* toxin levels. Toxin titer was influenced by background microbiota composition (Fig. 12B), with KPN MH189, *P. mirabilis* #2 and VRE colonization prior to *C. difficile* infection resulting in significantly higher toxin levels in cecal contents, and *B. sartorii* colonized mice having reduced levels.

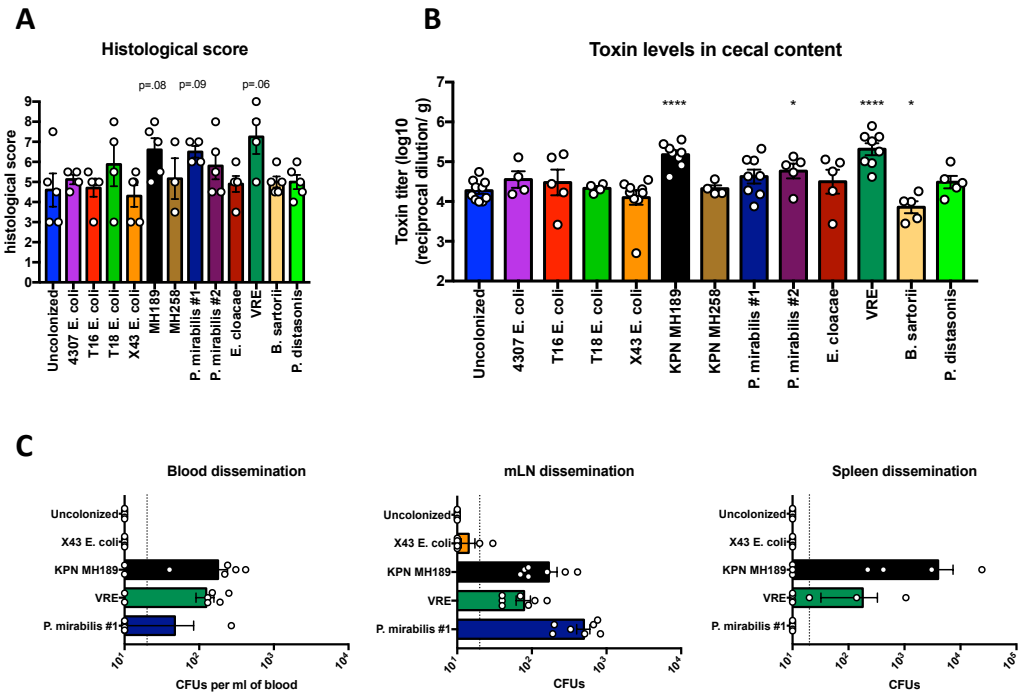

Figure 12: Residual microbiota impact on host pathology, cecal toxin levels, and bacterial dissemination during CDI

(A) Pathology score of histological tissue sections based on cellular infiltration, edema (host response), and epithelial layer damage **(B)** C. difficile toxin in cecal content was determined by an in vitro cytotoxicity assay **(C)** Mice (n=7 from two independent experiments) colonized with one of four strains were treated with ampicillin, inoculated with one of X43 *E. coli*, KPN MH189, VRE, or *P. mirabilis* #1 with an accompanying unreconstituted control group. Mice were inoculated with 200 spores of *C. difficile* VPI 10463 and euthanized on day 2 post *C. difficile* infection. Blood, mesenteric lymph nodes (mLN), and spleen were isolated and homogenized and bacterial dissemination post *C. difficile* infection was determined via selective plating. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data shown are ± SEM.

To determine if epithelial damage caused by *C. difficile* enhances dissemination of antibiotic-resistant bacterial strains, we quantified CFUs in tissues on day 2 post *C. difficile* VPI 10463 inoculation in mice colonized with X43 *E. coli, K. pneumoniae* MH189, VRE, or *P. mirabilis* #1 (Fig. 12C). *K. pneumoniae* MH189, VRE, and *P. mirabilis* #1 disseminated to blood and mesenteric lymph nodes, and viable *K. pneumoniae* MH189 and VRE were

detected in spleens. X43 *E. coli* did not disseminate except in low abundance to the mesenteric lymph nodes. Our results demonstrate that the residual microbiota influences the inflammatory response to *C. difficile* infection and that *C. difficile* infection is associated with greater dissemination of some bacterial strains that persist in the cecum and colon.

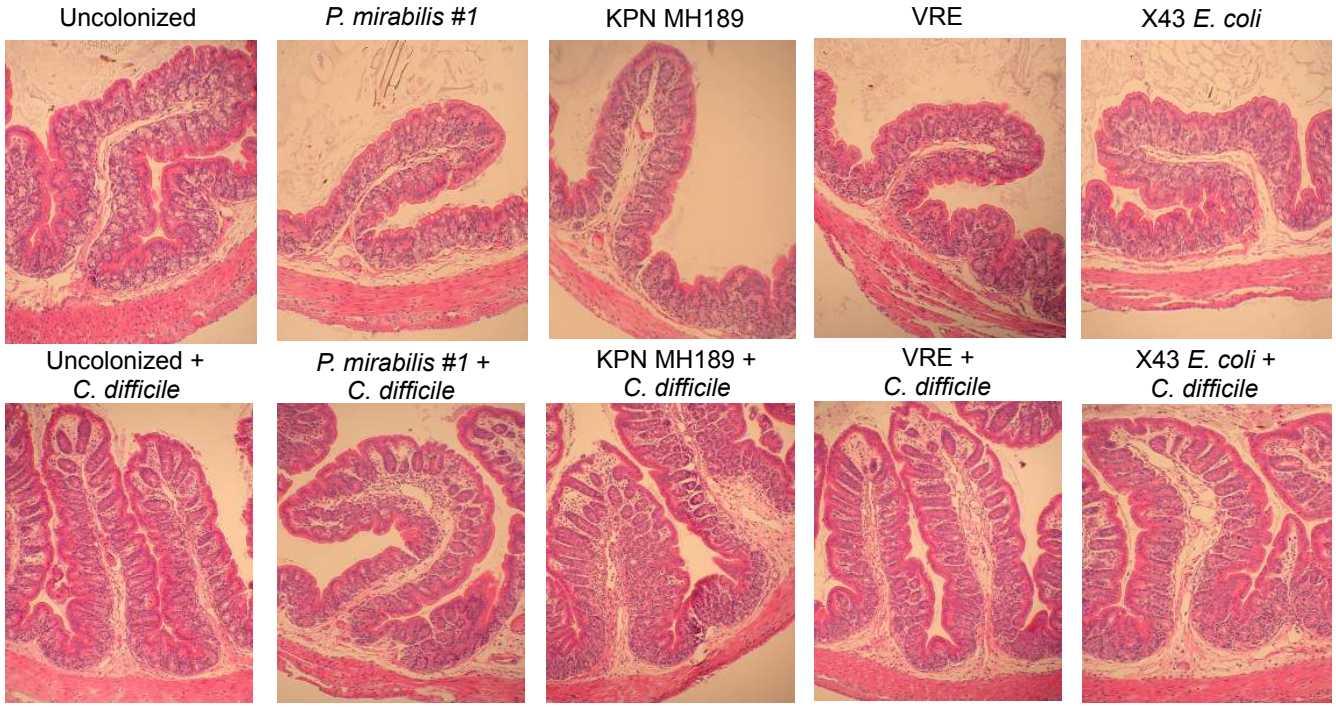

Figure 13: Residual microbiota did not impact tissue histology following CDI

C57BL/6 mice were infected with 200 spores of *C. difficile* (VPI 10463 strain) following antibiotic pretreatment and euthanized 2 days later. Pictured are representative H&E-stained cecal sections from antibiotic-treated mice colonized with different ampicillin-resistant bacterial strains in the presence or absence of *C. difficile* infection.

2.8 Discussion

Antibiotic treatment can deplete commensal bacterial populations that provide colonization resistance against bacterial pathogens, including highly antibiotic-resistant bacterial strains that are important causes of infection in

hospitalized patients. Loss of colonization resistance can result in the marked expansion of antibiotic-resistant strains of *E. faecium*, *E. coli*, *K. pneumoniae* and many of strains belonging to the Enterobacteriaceae family. Although intestinal domination by these strains is associated with a markedly increased risk of bacteremia and sepsis, particularly in the setting of cancer treatment with hematopoietic stem cell transplantation (Taur et al., 2012), the impact of domination on the mucosal immune response is incompletely defined. A previous study demonstrated that VRE and *K. pneumoniae* differ with respect to their ability to penetrate the dense mucus layer and access MLNs (Caballero et al., 2015), suggesting that immune activation by antibiotic-resistant bacterial strains likely varies. Our results confirm this notion by demonstrating that induction of inflammatory cytokines can differ between bacterial strains. The mechanisms by which dominating bacterial strains traverse the intestinal epithelium and the pathways involved in immune activation remain to be defined.

The balance between inflammatory and regulatory immune responses influences the initiation, progression and resolution of colitis. Inflammatory responses in the gut are generally provoked by intestinal microbes and their molecular products, which can differ dramatically in terms of composition. We tested the hypothesis that the microbiota composition in the intestine during the course of *C. difficile* infection would alter the severity of colitis and potentially alter recovery. We chose to introduce antibiotic-resistant bacterial species that commonly reside in the intestine, including *K. pneumoniae*, *E. coli*, *E. cloacae*, *P. mirabilis* and also two commensal bacterial species belonging to the

bacteroidetes phylum. Surprisingly, we did not find that these bacterial strains significantly altered the course of *C. difficile* infection.

Previous studies have demonstrated that a small subset of commensal bacterial strains can mediate resistance against *C. difficile* infection. Mechanisms of commensal-mediated resistance include production of secondary bile acids by anaerobic strains such as *Clostridium scindens* (Buffie et al., 2015) and also depletion of nutrients that support *C. difficile* growth (Ng et al., 2013). The ampicillin-resistant bacterial strains that we introduced into antibiotic-treated mice do not convert primary to secondary bile and apparently do not deplete nutrients that are essential for *C. difficile* virulence. Thus, we did not detect an impact of these strains on colitis associated with *C. difficile* infection.

Our collection of antibiotic-resistant bacterial strains was limited and it is possible that other bacterial strains, perhaps those expressing potential enterotoxins, could enhance *C. difficile* colitis. Certain strains of *Bacteroides fragilis*, for examples, express an enterotoxin that has been associated with the development of colon cancer (Wu et al., 2009). Determining whether such strains can enhance *C. difficile* colitis will require further investigation. Our results, however, suggest that in most settings, the residual microbiota is unlikely to provide an explanation for the range of colitis severities seen in patients with *C. difficile* infection. Differences in the virulence of *C. difficile* strains and host factors such as immune competence are the most significant determinants of colitis severity during *C. difficile* infection.

CHAPTER 3

MONOCYTE AND NADPH OXIDASE DEFICIENT MICE EXHIBIT SYSTEMIC INFECTION AFTER DENSE INTESTINAL COLONIZATION WITH KLEBSIELLA PNEUMONIAE

3.1 Introduction

One of the critical functions of a healthy intestinal microbiota is to maintain colonization resistance against pathogens and pathobionts. In healthcare settings, antibiotic administration can disrupt the intestinal microbiota, allowing for robust expansion of Enterobacteriaceae within the intestine, and among them, *Klebsiella pneumoniae* (Kp). While some enteric infections like *C. difficile* can cause gastroenteritis, Kp can reach very high density in the intestine without triggering overt inflammation or pathology. For Kp and other Enterobacteriaceae, the central clinical problem is that intestinal expansion greatly increases the risk for subsequent manifestation of a bloodstream infection (BSI) in vulnerable patient populations. Patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HCT) are highly immunocompromised, such that enteric domination by Kp within this patient population leads to a significant risk of developing a BSI (Taur et al., 2012). Kp producing the KPC-type carbapenemase have emerged as a widespread cause of multidrug-resistant nosocomial infections, making these highly antibiotic resistant strains increasingly difficult to treat.

There is currently minimal understanding of how Kp, despite not inducing overt inflammation, is able mechanistically to traverse the intestinal epithelial

barrier and enter the bloodstream. However, the observation that BSI is associated with allo-HCT patients and not immunocompetent patients that have lost colonization resistance suggests that systemic dissemination may be governed in part by the host immune response, or lack thereof.

Monocytes are bone-marrow residing leukocytes that can rapidly traffic into the bloodstream and circulate to infected or inflamed tissues. These cells are pluripotent, and depending on the inflammatory environment that they enter, they can acquire phenotypes that range from immunosuppressive IL-10 production to pro-inflammatory TNF and iNOS production (Serbina et al., 2003); (Morhardt et al., 2019). A defining feature of resident gut macrophages is their high replenishment rate from blood monocytes attributed to tonic commensal stimulation of the intestine (Bain et al., 2014). In contrast, almost all other tissues contain locally maintained macrophage populations, which coexist with monocyte-replenished cells at homeostasis. Rapid monocyte recovery following allo-HCT is correlated with improved overall survival (Le Bourgeois et al., 2016), and our lab has previously found that monocytes play a crucial role in controlling lung Kp infections. Therefore, we hypothesized that monocytes could be playing a critical role in preventing Kp systemic dissemination from the intestine. We used a mouse model in which we could deplete the monocyte compartment in mice and determined that monocyte depletion leads to significant dissemination of Kp from the intestine of Kp dominated mice. Through the use of additional testing in several immunodeficient mouse backgrounds, we determined that Cybb ($gp91^{phox}$) $^{-/-}$ mice recapitulate the same phenotype, suggesting that

monocytes' ability to perform intracellular killing on Kp can prevent or contain systemic dissemination from the intestine.

3.2 Murine intestine can become stably dominated by K. pneumoniae

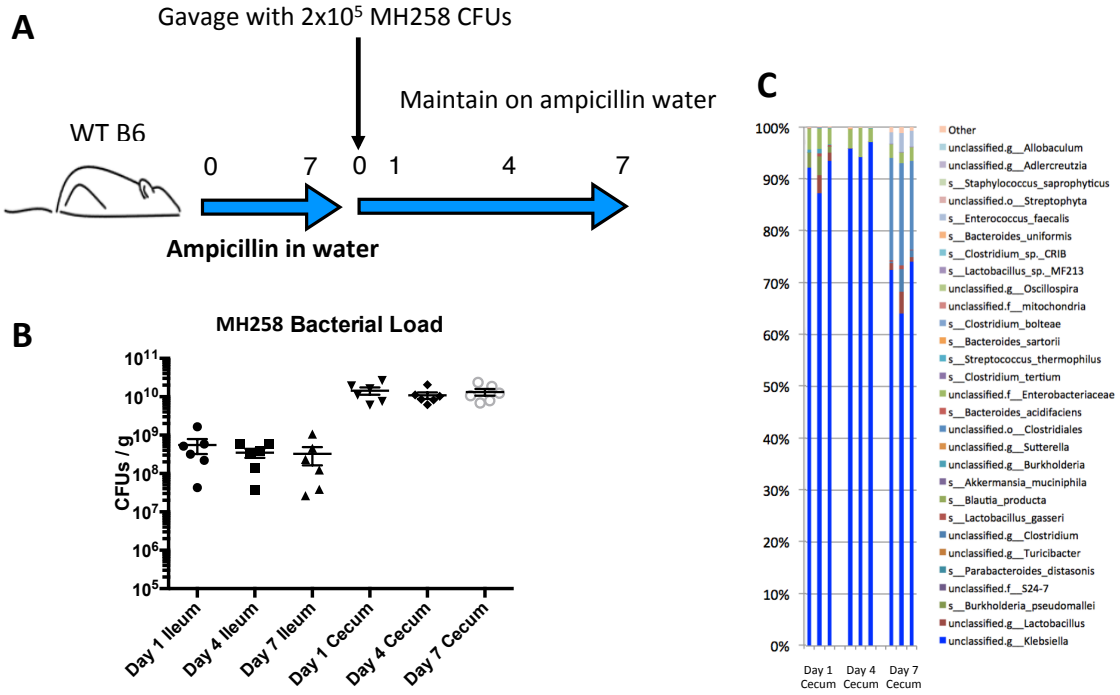

Figure 14: Mouse microbiota can be stably dominated by *K. pneumoniae* strain MH258

(A) Wildtype C57BL/6 mice were treated with ampicillin (0.5g/L) to be consumed ad libitum in drinking water, then inoculated with MH258. (B) Ileal and cecal content was selectively plated and CFUs were quantified on day 1, 4, and 7 post colonization. (C) 16s rRNA was purified and sequenced, revealing bacterial composition by percentage within the cecal content. Results compiled from 2 independent experiments (n= 9 mice per experiment, n=6 total mice per group).

Treatment with ampicillin, a broad-spectrum antibiotic, renders C57BL/6 mice from Jackson Laboratories highly susceptible to dense colonization with Kp. We inoculated mice after one week of ampicillin treatment with Kp strain MH258 (Fig 14A), which expresses carbapenemase and thus represents the emerging

group of highly antibiotic-resistant strains. This resulted in dense colonization of the ileum and cecum, with approximately 10^{10} colony-forming units (CFU) per gram of feces detectable in cecal content after selective plating (Fig 14B). Colonization with Kp was rapid and stable, with mice reaching over 90% domination of the intestinal microbiota by Kp one day post inoculation, and maintaining similar levels both on day 4 and 7 post inoculation as measured by 16s rRNA abundance in cecal content (Fig 14C). Mice that are densely colonized with *K. pneumoniae* do not lose weight or display any morbidity or pathology compared to mice that are untreated or mice that only receive ampicillin.

3.3 K. pneumoniae colonization does not significantly affect monocyte recruitment to the intestinal lamina propria but leads to higher blood monocyte levels

To determine the kinetics of inflammatory cell recruitment to the intestine following dense colonization with Kp, we quantified inflammatory monocytes ($Ly6C^{hi}$) and neutrophils ($Ly6G^{+}$) by flow cytometry in the small intestinal lamina propria (SILP), large intestinal lamina propria (LILP), bone marrow, and blood of mice at different time points following dense colonization with Kp. These monocyte frequencies were compared to those from mice that were either not administered antibiotics or mice that received only antibiotics and not Kp. We did not observe significant fluctuations to neutrophil levels in any compartment (data not shown). We observed no significant differences in monocyte levels within the SILP and LILP before or after Kp colonization (Fig 15A and B). However, we observed a statistically insignificant, but detectable increase in blood monocyte levels day 1 post colonization with Kp, suggesting increased trafficking from the

bone marrow into circulation (Fig 15C). Higher levels of blood monocytes might result from Kp entering the bloodstream in low abundance. Interestingly, we did not observe a concomitant decrease monocyte frequencies in the bone marrow (Fig 15D), suggesting that monocytopoiesis may increase in the bone marrow while monocytes are infiltrating the bloodstream.

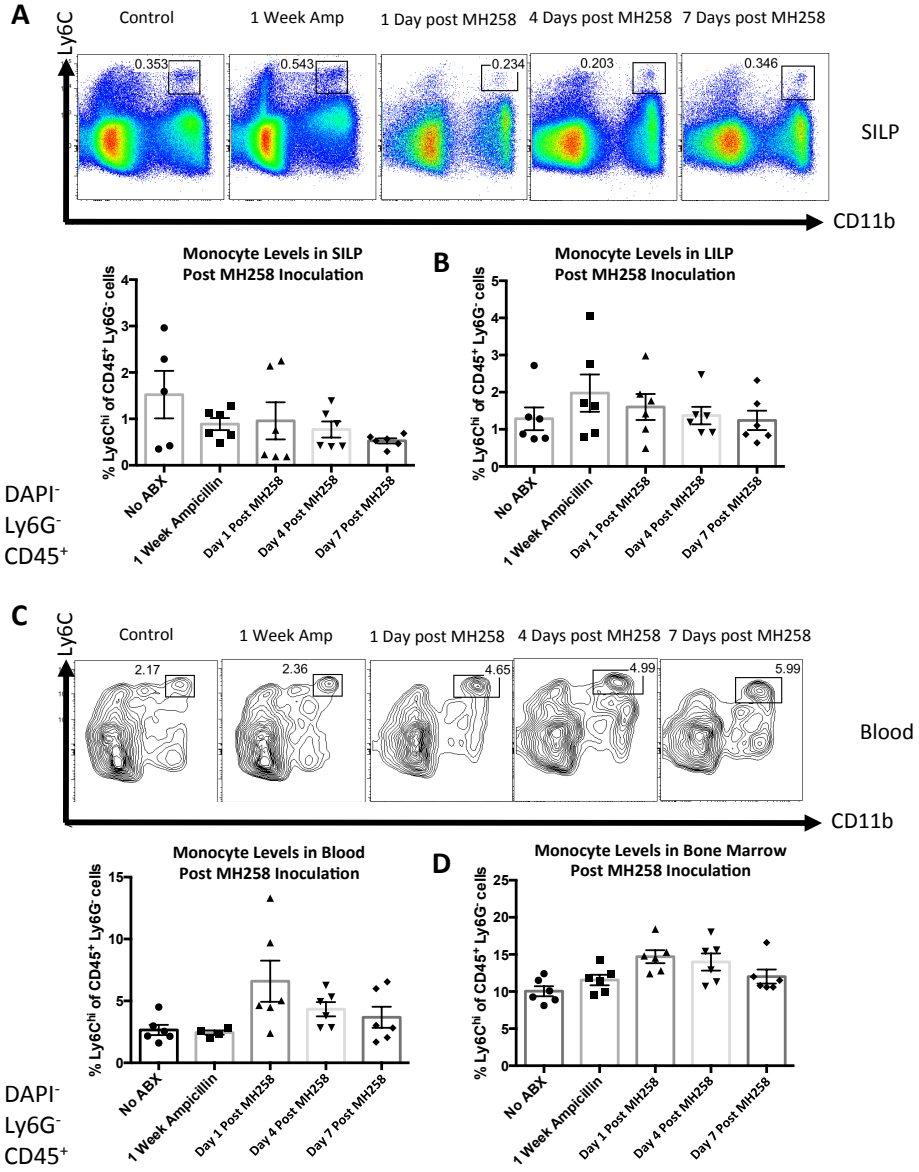

Figure 15: *K. pneumoniae* colonization does not significantly affect monocyte recruitment to the intestinal lamina propria but leads to higher blood monocyte levels

(A) WT C57BL/6 mice were treated with ampicillin and colonized with MH258. For comparison, one group was untreated (No ABX), and another group was administered ampicillin but not colonized (1 Week Ampicillin). On the corresponding day, the SILP, (B) LILP, (C) Blood, and (D) Bone Marrow were isolated and flow cytometry was performed to examine monocyte and neutrophil infiltration. Results compiled from 2 independent experiments (n= 15 mice per experiment, n=6 total mice per group).

3.4 Depletion of monocytes in K. pneumoniae dominated mice significantly increases systemic bacterial dissemination from the intestine

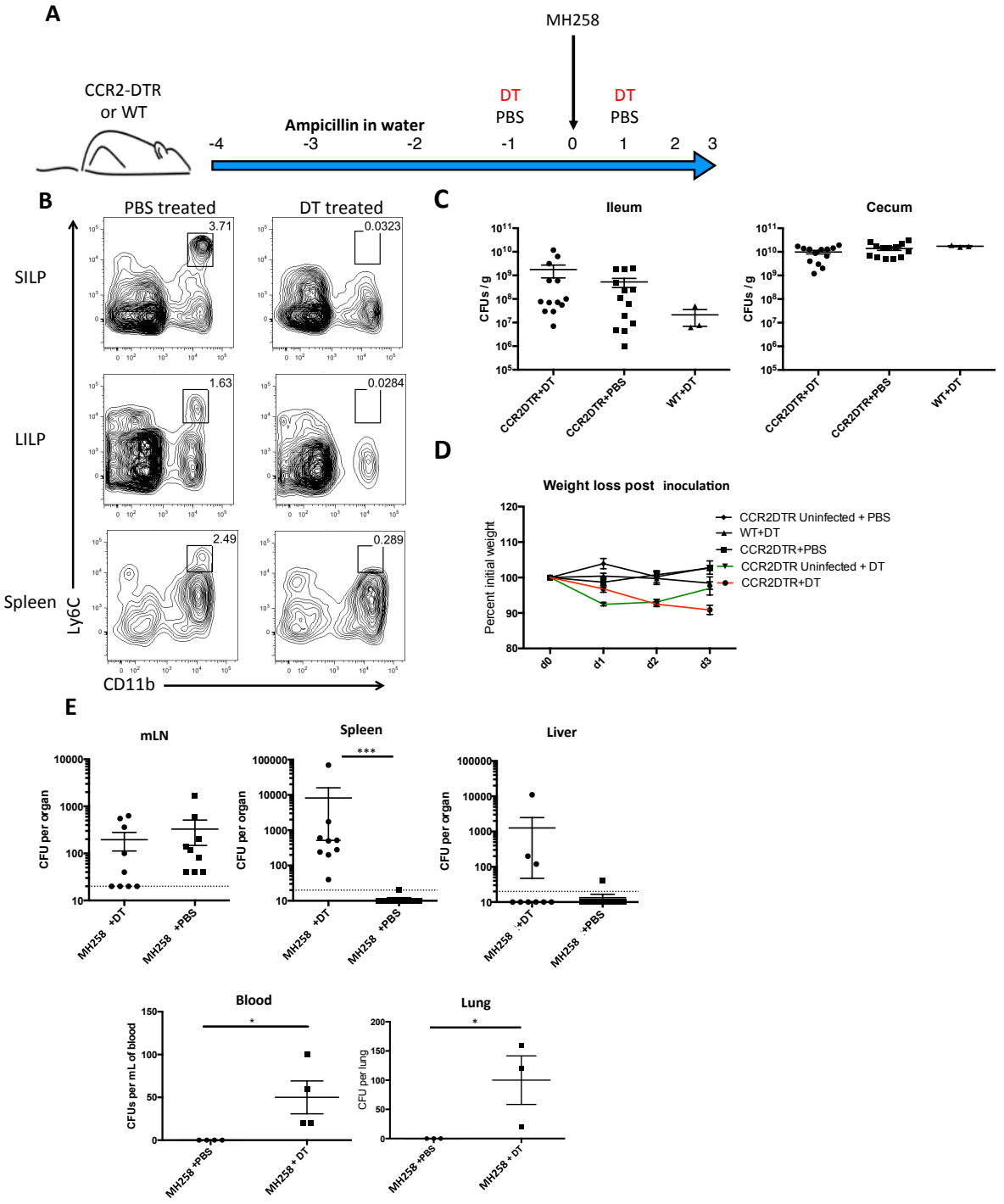

Figure 16: Monocyte depletion significantly increases systemic bacterial dissemination from the intestine

(A) Experimental schematic (B) Flow cytometry performed to verify successful systemic monocyte depletion following two doses of DT treatment in CCR2-DTR mice (C) Selective plating within both the ileal and cecal contents of mice colonized with *K. pneumoniae* strain MH258 (D) Weight loss post inoculation by group (E) Selective plating of the mLN, spleen, liver, blood, and lung for *K. pneumoniae* strain MH258 CFUs. Results compiled from 3 independent experiments (n= 9-10 mice per experiment, n = 9 total for all groups except DT toxicity control group which n=3).

To determine the contribution of monocytes to bacterial clearance from the lungs, we depleted $CCR2^{+}$ monocytes by administering diphtheria toxin (DT) to CCR2-DTR mice (Fig 16A). We observed that two doses of DT were sufficient to deplete monocytes in the SILP, LILP, spleen, and blood among CCR2-DTR mice (Fig 16B). All mice were treated with ampicillin prior to inoculation with Kp*,* except for control groups that were uncolonized with Kp*.* There were no differences in Kp colonization between groups, with that received Kp becoming similarly densely colonized (Fig 16C). There was considerable weight loss in mice that were monocyte depleted and also dominated with Kp in contrast with mice that were monocyte depleted but did not receive Kp*,* which lost weight temporarily but regained it by day 3 post inoculation (Fig 16D).

In order to determine if monocyte depletion led to significant differences in the manifestation of systemic dissemination from the intestine, we cultured mesenteric lymph nodes, spleen, liver, and blood from CCR2-DTR mice dominated by Kp that either received DT or not. In Kp dominated mice, monocyte depletion did not affect dissemination from the intestine to the mesenteric lymph nodes. However, there was a significant increase in systemic Kp dissemination to

the spleen and bloodstream, and multiple instances of substantial dissemination to the liver and lung as well (Fig 16E). However, this dissemination is not a function of colonization density, as there was no significant correlation between disseminated CFUs and total number of CFUs in the cecum, where Kp is most abundant in the intestine (data not shown). These data suggest that monocytes are playing an important role in preventing Kp dissemination from the intestine, as only when monocytes are depleted does detectable systemic dissemination from the intestine take place.

3.5 Intestinal domination by K. pneumoniae leads to increased splenic monocyte and neutrophil infiltration

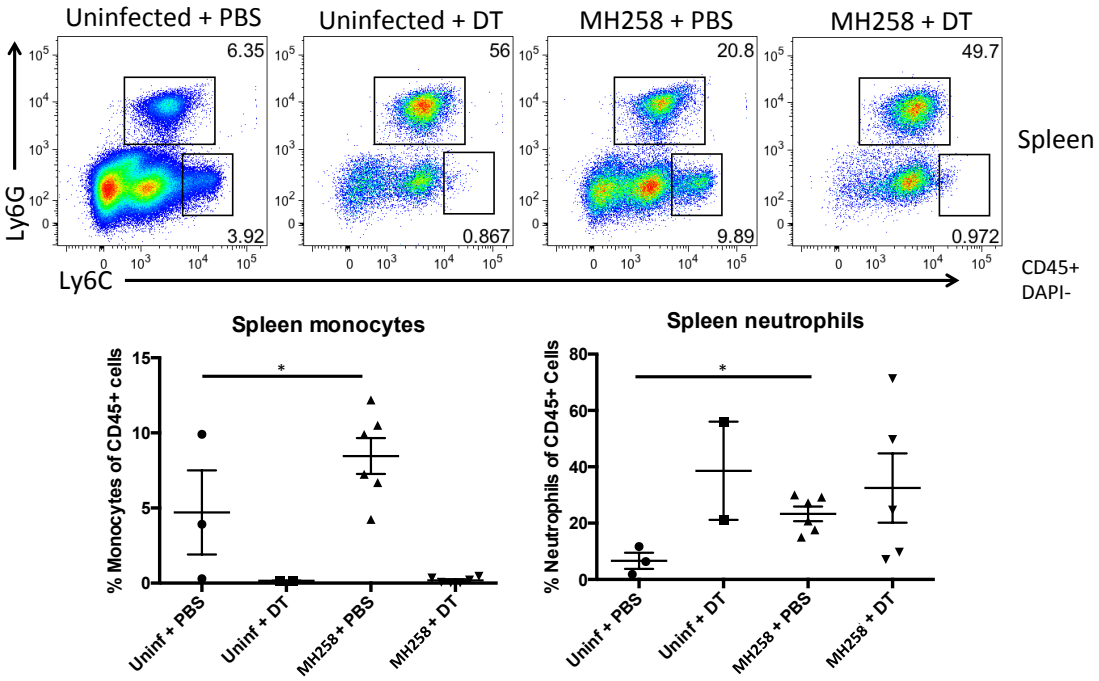

Figure 17: Intestinal domination by *K. pneumoniae* leads to increased splenic monocyte and neutrophil infiltration

Flow cytometry performed on spleen of CCR2-DTR mice that were treated with ampicillin and then colonized with *K. pneumoniae* strain MH258. Top panel shows representative plots after filtering for live, $CD45^+$ splenocytes. Bottom panels show quantification of monocyte and neutrophil infiltration on the basis of

Ly6C^{hi} and Ly6G^{+} expression, respectively. Results compiled from 2 independent experiments (n= 7-8 mice per experiment, n= 6 total mice per group except uninfected groups which n=2-3).

Based on the fact that we observed increased monocyte levels in the blood as soon as one day after Kp domination, we hypothesized that perhaps Kp disseminates at low levels from the intestine into the bloodstream that might only be detectable when monocytes are depleted due to monocytes normally killing these live disseminated bacteria. We examined monocyte and neutrophil levels within the spleen of mice that either received Kp or not and either were monocyte depleted or not. We observed an increase of splenic monocytes and neutrophils in Kp dominated wildtype mice compared to uninfected wildtype mice (Fig 17). There was also a substantial compensatory increase in neutrophil abundance when monocytes are depleted, which could be a byproduct of rapid monocyte depletion in CCR2-DTR mice. However, these data suggest that monocytes are necessary to reduce live Kp systemic dissemination. This supports the hypothesis that there is low-level dissemination that occurs at steady state when murine microbiota is dominated by Kp*,* only for monocytes to facilitate their destruction when the monocyte compartment is intact*.* However, we cannot rule out the possibility that other cells are also playing a role in Kp clearance within distal tissues.

3.6 K. pneumoniae ability to disseminate from the intestine differs in mice with distinct forms of immunodeficiency

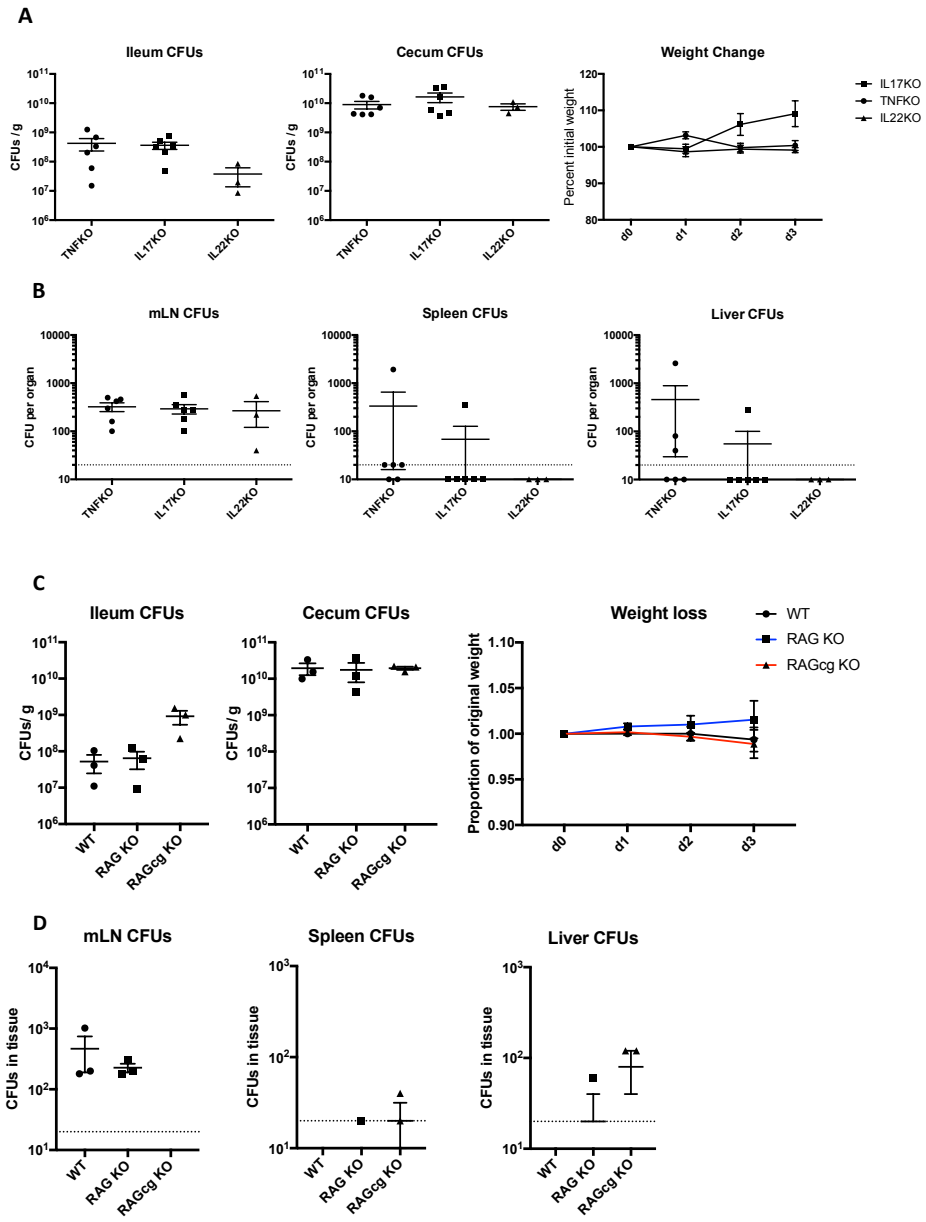

Figure 18: *K. pneumoniae* ability to disseminate from the intestine differs in mice with distinct forms of immunodeficiency

(A) Selective plating of Ileal and cecal CFUs day 3 post inoculation of *Tnfα*$^{-/-}$, *Il17*$^{-/-}$, *Il22*$^{-/-}$ mice, as well as their weight loss post inoculation (B) Selective plating of the mLN, spleen, liver, blood, and lung for *K. pneumoniae* strain MH258 CFUs. (C) Selective plating of Ileal and cecal CFUs day 3 post inoculation of WT, *Rag*$^{-/-}$, *Ragγc* $^{-/-}$ mice, as well as their weight loss post inoculation. (D) Selective plating of the mLN, spleen, liver, blood, and lung for *K. pneumoniae* strain MH258 CFUs. Results compiled from 2 independent experiments (n= 9-10 mice per experiment, n=3 or 6 mice total per group in A and B, n=3 mice total per group in C and D).

After determining that monocytes play a significant role in preventing Kp dissemination from the intestine, we hypothesized that monocytes or monocyte-derived cells are responsible for Kp clearance within distal tissues, or monocytes are acting to enhance the phagocytic capabilities of other cells. In order to determine if monocytes were singularly important in the prevention of Kp dissemination, we treated a number of mice with distinct immune deficient mouse genetic backgrounds with ampicillin and inoculated them with Kp to see if dissemination could be recapitulated.

Our lab previously determined that monocyte derived TNF-α potentiates ILC3s in the lung to produce IL-17 in lung Kp infections, with all of these components proving critical for effective Kp clearance. We tested to see if *Tnfa*$^{-/-}$, *Il17*$^{-/-}$, *Il22*$^{-/-}$ mice when treated with ampicillin and intestinally dominated with Kp also exhibit dissemination from the intestine into distal tissues. We observed that while these different mouse backgrounds were capable of being densely colonized with Kp, there was reduced dissemination compared to monocyte depletion. *Tnfa*$^{-/-}$ mice displayed some dissemination to the spleen and liver, but at lower levels and CFU counts than when monocytes are depleted (Fig 18A and B). We also examined the capacity for Kp dissemination in *Rag*$^{-/-}$ and *Ragγc*$^{-/-}$ mice. We found that again, despite the mice being very densely colonized in both the ileum and cecum, the dissemination phenotype that took place when monocytes were depleted was only partially recapitulated, as not all mice had detectable CFUs within the spleen (Fig 18C and D). However, increased dissemination in *Ragγc*$^{-/-}$ mice compared to *Rag*$^{-/-}$ mice suggests that ILCs might

be playing a contributing role to preventing or containing Kp dissemination, though the mechanism of Kp clearance differs from lung infection based on these data.

3.7 NADPH oxidase deficiency phenocopies K. pneumoniae dissemination observed in monocyte depleted mice

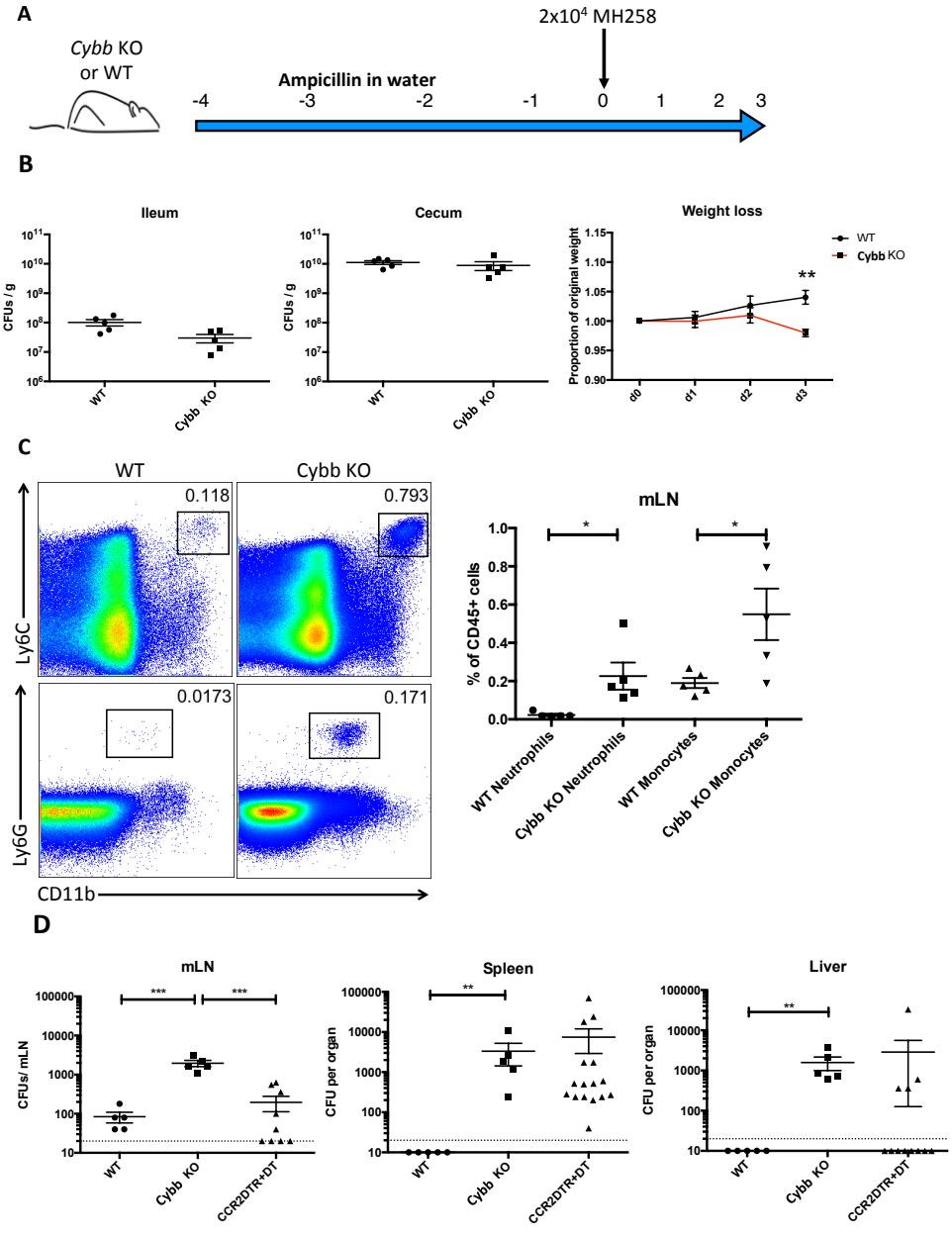

Figure 19: NADPH oxidase deficiency phenocopies *K. pneumonia* dissemination in monocyte depleted mice

(A) Experimental schematic (B) Selective plating of Ileal and cecal CFUs day 3 post inoculation of WT and *Cybb* ($gp91^{phox}$)$^{-/-}$ mice, as well as their weight loss post inoculation (C) Representative flow cytometry plots of monocyte and neutrophil infiltration into the mLNs and the summarized results (D) Selective plating of the mLN, spleen, and liver for *K. pneumoniae* strain MH258 CFUs compared to levels of dissemination in monocyte depleted CCR2-DTR mice that were colonized with *K. pneumoniae* strain MH258 (n=5 mice per group except n=16 for CCR2-DTR mice plus DT in (D))

In order to assess whether *K. pneumoniae* dissemination is a function of impaired intracellular killing, we tested to see if *Cybb* ($gp91^{phox}$)$^{-/-}$ mice, which are lacking a critical component of the NOX2 complex responsible for generating reactive oxygen species (ROS), can phenocopy monocyte depletion for *K. pneumoniae* dissemination. After cohousing the *Cybb* ($gp91^{phox}$)$^{-/-}$ with wildtype mice to normalize their microbiota, we administered ampicillin and inoculated with *K. pneumoniae* (Fig 19A). Both groups of mice were similarly colonized, but the *Cybb* ($gp91^{phox}$)$^{-/-}$ mice lost a significant amount of weight by day 3 post inoculation (Fig 19B). Additionally, the *Cybb* ($gp91^{phox}$)$^{-/-}$ mice had significantly higher mLN dissemination, significantly higher monocyte and neutrophil infiltration into the mLN, and a significant dissemination of CFUs into the spleen and liver (Fig 19C and D). Given that monocyte depletion and *Cybb* ($gp91^{phox}$)$^{-/-}$ mice have nearly identical phenotypes for dissemination when dominated with *K. pneumoniae,* it suggests that intracellular killing by monocytes is a key component of containment of disseminated Kp.

3.8 Discussion

Dense colonization of the intestine by hospital-associated, extended-spectrum antibiotic resistant strains of Kp is a major clinical concern because of

the pronounced increased risk it entails for disseminated infection as well as spread to uninfected patients (Taur et al., 2012). Kp strain MH258 belongs to the sequence type 258 (ST258), which is now the dominant strain in the United States that is carbapenem resistant, and has spread to many other countries (Chen et al., 2014). Previous work shows that monocytes, but not neutrophils, are essential for clearance of strain MH258 in the setting of lung infections (Xiong et al., 2016). Additionally, absolute monocyte recovery is correlated with improved hematopoietic cell transplant outcomes (Le Bourgeois et al., 2016) and patients who receive allo-HCT are one of the most at risk patient populations for being intestinally dominated by Kp. Here, we provide evidence that monocytes play an important role in preventing Kp systemic dissemination to distal sites in mice after being densely colonized with Kp following antibiotic treatment.

The intestinal mucosa has a polarized monolayer of enterocytes covered in a mucus layer and protected by host-secreted antimicrobial peptides that isolates the host from luminal microbes and antigens. It remains unclear as to how Kp is able to translocate across the epithelium, though it has been suggested that some clinical Kp strains can employ a transcellular pathway to translocate into the underlying tissue (Hsu et al., 2015), which have also been suggested for other Enterobacteriaceae like *E. coli* and *Proteus mirabilis (Wells and Erlandsen, 1991).* In our work, there does seem to be a baseline level of dissemination that occurs regardless of whether monocytes have been depleted, as mice dominated by Kp always have detectable CFUs in the mLNs, and show a significant increase in monocyte and neutrophil abundance in the spleen of

wildtype mice, which is the biggest reservoir for dissemination when monocytes are depleted. Previous work shows that strain MH258 is able to infiltrate the mucus layer while inducing mucus thickening after antibiotic induced mucus layer thinning occurs, though it is unclear whether enhanced mucus production is a result of host immune stimulation due to bacterial translocation (Caballero et al., 2015). The Kp capsule, an extracellular polysaccharide structure that protects Kp from lethal serum factors and phagocytosis is also a factor that could help to explain how Kp is able to translocate across the epithelium (Sahly et al., 2000). It would be valuable to examine whether the dissemination phenotype we observed also manifests when using different clinical strains of Kp that have different capsular composition and more reliance on neutrophils rather than monocytes for bacterial clearance, traits which have been examined *in vivo* in other contexts (Xiong et al., 2015).

Normally, commensals and pathogens that breach the epithelial barrier are engulfed, killed, processed, and presented by an array of mononuclear phagocyte populations and polymorphonuclear leukocytes/granulocytes. The antimicrobial arsenal of these cells includes proteases and reactive oxygen species produced by the NADPH oxidase complex, of which CYBB is a major component (Panday et al., 2015). NADPH deficiency is established as causing susceptibility to bacterial infection and inflammatory disease, and is referred to as chronic granulomatous disease (CGD), and a majority are caused by mutations to the *Cybb* gene (Holland, 2010). Our data suggests that a defect in the ability to perform intracellular killing of Kp has the same dissemination and weight loss

that monocyte depletion, suggesting that either monocytes are the primary phagocytes responsible for preventing systemic dissemination, or monocytes are potentiating the phagocytic capacity of neutrophils or another cell subset. RNA-seq analysis comparing Kp colonized colonic tissue that was monocyte depleted to Kp colonized colonic tissue that is replete with monocytes revealed that *Cybb* had significantly lower expression when monocytes were depleted (data not shown), which suggests that monocytes have robust CYBB expression in colonic tissue colonized by Kp. Our findings collectively suggest that monocytes play an underappreciated role in preventing Kp dissemination from the intestine, though it still remains unclear whether this is a finding that can be applied to a wide variety of Kp strains and Enterobacteriaceae as a whole, or whether this only applies to the MH258 strain of Kp.

DISCUSSION

The microbiota can act as a double-edged sword in the setting of enteric infection, as resident bacteria can provide beneficial effects to the host (Kamada et al., 2013), including bacterial metabolites such as vitamins and short chain fatty acids or deleterious effects in the setting of inflammation and/or epithelial damage. At the same time, commensal species help support the maintenance of several barriers, including the mucus layer and production of antimicrobial peptides which limit contact between microbes and the host immune system and contribute to gut homeostasis (Buffie and Pamer, 2013). A role for the microbiota in eliciting intestinal inflammation is supported by findings that chemical-induced and spontaneous colitis are reduced or prevented in antibiotic-treated mice and germ-free mice (Garrett et al., 2007) (Kirkland et al., 2012) (Vijay-Kumar et al., 2007). In addition, mouse models have revealed that *Bacteroides* species and members of *Enterobacteriaceae* family including *Klebsiella pneumoniae* and *Proteus* mirabilis can promote colitis (Bloom et al., 2011) (Garrett et al., 2010). We are only beginning to appreciate the influence that individual microbial taxa can have on the host immune response, both when the epithelium is intact (Geva-Zatorsky et al., 2017), much less when the epithelium is disrupted, in which microbial molecules are able to traverse the epithelial barrier, driving certain host immune pathways into overdrive (Seo et al., 2015).

Antibiotic treatment can deplete commensal bacterial populations that provide colonization resistance against bacterial pathogens, including highly antibiotic-resistant bacterial strains that are important causes of infection in

hospitalized patients. Loss of colonization resistance can result in the marked expansion of antibiotic-resistant strains of *E. faecium*, *E. coli*, *K. pneumoniae* and many strains belonging to the Enterobacteriaceae family. Although intestinal domination by these strains is associated with a markedly increased risk of bacteremia and sepsis, our work in chapter two sought to better define the impact of domination on the mucosal immune response. A previous study demonstrated that VRE and *K. pneumoniae* differ with respect to their ability to penetrate the dense mucus layer and access MLNs (Caballero et al., 2015), suggesting that immune activation by antibiotic-resistant bacterial strains likely varies. We confirmed this notion by demonstrating that induction of inflammatory cytokines can differ between bacterial strains. The mechanisms by which dominating bacterial strains traverse the intestinal epithelium and the pathways involved in immune activation remain to be defined, and should be investigated further. Pinpointing exactly which cell subsets are most influential in these settings would be a good starting point.

The balance between inflammatory and regulatory immune responses influences the initiation, progression and resolution of colitis. Inflammatory responses in the gut are generally provoked by intestinal microbes and their molecular products, which can differ dramatically in terms of composition. We tested the hypothesis that the microbiota composition in the intestine during the course of *C. difficile* infection would alter the severity of colitis and potentially alter recovery. We chose to introduce antibiotic-resistant bacterial species that commonly reside in the intestine, including *K. pneumoniae*, *E. coli*, *E. cloacae*, *P.*

mirabilis and also two commensal bacterial species belonging to the bacteroidetes phylum. Surprisingly, we did not find that these bacterial strains significantly altered the course of *C. difficile* infection.

Our collection of antibiotic-resistant bacterial strains was limited and it is possible that other bacterial strains, perhaps those expressing potential enterotoxins, could enhance *C. difficile* colitis. Determining whether such strains can enhance *C. difficile* colitis will require further investigation. Our results, however, suggest that in most settings, the residual microbiota is unlikely to provide an explanation for the range of colitis severities seen in patients with *C. difficile* infection. Differences in the virulence of *C. difficile* strains and host factors such as immune competence are the most significant determinants of colitis severity during *C. difficile* infection.

One unexpected finding was that the residual microbiota after antibiotic treatment significantly influenced *C. difficile* toxin titers, particularly when mice were densely colonized with KPN MH189 and VRE. It is currently unknown whether this is due to direct bacteria-bacteria interactions, or whether the mechanism is indirect, through inducing expression of a host-derived factor that alters *C. difficile* toxin production. Toxin levels have long been thought to be correlated with the severity of CDI (Akerlund et al., 2006) (Wren et al., 1987). This is reinforced by the emergence of the hypervirulent NAP1/027 strain in the early 2000's, which is notable for its increased toxin A and B production compared to non-epidemic strains (Warny et al., 2005). Indeed, recent work from our lab (Lewis et al., 2017) found a strong correlation between toxin levels and

disease severity when examining 33 different *C. difficile* isolates using a similar murine model as this study. Additionally, higher levels of pathology in the KPN MH189 and VRE dominated mice might partially account for some of the higher toxin titers, given that increased epithelial cell death could mean fewer toxin receptors available and more unbound toxin in the lumen. However, it is unlikely this would account for over a 10-fold increase in toxin titers, as the epithelial damage was only slightly worse in these mice.

C. difficile toxin production has been shown to be influenced by growth conditions and environmental factors like nutritional signals, as reduced concentrations of biotin or increased levels of short-chain fatty acids can increase toxin production, while some sugars and amino acids in media conditions can substantially reduce toxin levels (Yamakawa et al., 1996) (Karlsson et al., 1999) (Karlsson et al., 2000) (Karlsson et al., 2008) (Bouillaut et al., 2013). It is already established that *C. difficile* toxin production can be regulated through bacteria-bacteria interactions through quorum sensing. *C. difficile* is capable of both inter- and intra-species communication that influences toxin production (Darkoh et al., 2015) (Martin et al., 2013) (Lee and Song, 2005), which also could be contributing to certain strains capacity to induce higher toxin levels.

Whether or not the significant increase in toxin production is induced through direct bacteria-bacteria communication or via a bacteria-host-bacteria mechanism is unclear, but investigating what bacterial derived factors influence this could lead to elucidation of potential therapeutic targets for controlling virulence factor expression in *C. difficile.*

In chapter three we provide evidence that monocytes play an important role in preventing Kp systemic dissemination to distal sites in mice after being densely colonized with Kp following antibiotic treatment. In our work, there does seem to be a baseline level of dissemination that occurs regardless of whether monocytes have been depleted, as mice dominated by Kp always have detectable CFUs in the mLNs, and show a significant increase in monocyte and neutrophil abundance in the spleen of wildtype mice, which is the biggest reservoir for dissemination when monocytes are depleted. It would be valuable to examine whether the dissemination phenotype we observed also manifests when using different clinical strains of Kp that have different capsular composition and more reliance on neutrophils rather than monocytes for bacterial clearance, traits which have been examined *in vivo* in other contexts (Xiong et al., 2015).

Our data suggests that a defect in the ability to perform intracellular killing of Kp results in the same dissemination and weight loss that monocyte depletion, suggesting that either monocytes are the primary phagocytes responsible for preventing systemic dissemination, or monocytes are potentiating the phagocytic capacity of neutrophils or another cell subset. Our findings collectively suggest that monocytes play an underappreciated role in preventing Kp dissemination from the intestine, though it still remains unclear whether this is a finding that can be applied to a wide variety of Kp strains and Enterobacteriaceae as a whole, or whether this only applies to the MH258 strain of Kp.

While the central role of CYBB is well established in antimicrobial defense, the identity of the cell types providing the NADPH oxidase dependent defenses is

still unclear. In addition to monocytes, neutrophils are well established as utilizing NADPH oxidase for antimicrobial defense purposes (Hapfelmeier et al., 2008), and we also cannot yet rule out a possible role for dendritic cell-mediated antigen presentation and T-cell priming (Savina et al., 2006). These possibilities should be investigated further. Additionally, we need to better determine the mechanism that activates NADPH oxidase in the mucosal phagocytes. Besides NADPH oxidase-deficiency/CGD, other primary immune deficiencies enhancing susceptibility to bacterial infection are deficiencies in Toll-like receptor- and IFNγ-R-signaling (van de Vosse et al., 2009), and testing the importance of these possible mechanisms would be worthwhile in the setting of Kp intestinal domination.

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