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Immune Activation by Novel Allobaculum Species Reveals Reciprocal Epistasis Among Human Gut Commensals

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Abstract

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Gut commensal microbes that elicit human immune responses are noteworthy for their ability to influence both local mucosal inflammation and, more rarely, systemic antibody responses. Here we isolated and characterized novel strains belonging to genus *Allobaculum* from inflammatory bowel disease (IBD) stool samples. In defined gnotobiotic mouse models we recapitulated the inflammatory effects of *Allobaculum sps.* and their notable induction of systemic immune responses at baseline. A microbial ecology screen revealed that this taxon is inversely correlated with *Akkermansia muciniphila*, and co-colonization experiments uncovered microbe-dependent redirection of immune phenotypes, which we term reciprocal epistasis. These immunostimulatory gut commensal strains exemplify the remarkable effects microbial ecology can have upon inflammation and immunity, as well as present a framework for unraveling the complexity of the gut microbiota with more mechanistic insight.

Immune Activation by Novel *Allobaculum* Species Reveals Reciprocal Epistasis Among Human Gut Commensals

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Dedication

This work is dedicated to Dr. George R. Borders of Stetson University and Palm Beach Atlantic University. His unfailing diligence, encouragement, and pursuit of excellence are exemplified in his adage, “Never give up, never give in, whenever you finish, you always win.”

Chapter 1. Characterization of human gut commensal *Allobaculum* species

1.1. Introduction

1.1.A. Human gut microbiota influences health, disease, and immune responses.

The world of microorganisms has fascinated scientists for nearly 500 years, despite the relatively recent technological advances allowing for visualization and precise examination of these tiny life forms. Hypotheses about spontaneous generation of germs in food products were first tested experimentally by the likes of Fracastoro (1546), Redi (1684), and later formalized by Pasteur (1859) (Richardson et al., 2020). The concept of microflora has been used in the medical community for over 100 years to describe the as-yet-undescribed forms of life dwelling within the human intestinal tract, and this terminology still lingers today. Meanwhile, microbiology as a discipline was forced to mature in the face of widespread bacterial dysentery and many other intestinal pathogens in the late 19th and early 20th centuries (Campbell, 1900; Goldman, 1924; Hewetson, 1904; Macharg, 1900), leading to clear findings that the microflora encompassed a complex mixture of bacteria, fungi, and viruses, but not plants, both in health and disease. In keeping with this, we employ the updated term “microbiota” to refer to the totality of all living microorganisms colonizing a given host and “microbiome” to refer to both the genetic content possessed by the microbiota and the research discipline more generally.

Broader appreciation of this host-associated microbial community has dramatically increased for the last 2-3 decades, reaching a fervor today that has captivated not only scientists but the health-conscious public. The wave of research that spurred this enthusiasm was largely propelled by the -omics technology boom that came around the turn of the 21st century. The ability to sequence genomes and transcriptomes at rapidly decreasing cost, and analyze vast data sets using amenable bioinformatics pipelines enabled widespread implementation of this technology to study microbial communities. A major advantage of this next generation sequencing approach is a culture-independent and unbiased examination of microbial communities that remain difficult, if not impossible, to culture in the laboratory.

As a result, the volume of human microbiota research has increased by several orders of magnitude and brought insights into assembly of microbial communities in infants (Dominguez-Bello et al., 2010), resistance against infections (van der Waaij et al., 1971), microbes of the skin (Grice et al., 2009), vagina (Ravel et al., 2011), respiratory tract (Charlson et al., 2011), and their influence on metabolic regulation (Turnbaugh et al., 2009), immune regulation (Atarashi et al., 2011; Bunker et al., 2015; Geva-Zatorsky et al., 2017; Ivanov et al., 2009; Kiner et al., 2021; Round and Mazmanian, 2010; Viladomiu et al., 2017; Xu et al., 2018), nervous system regulation (Bravo et al., 2011), and a near-infinite list of studies implicating the microbiota in many disease states. Bacteria are both ubiquitous and diverse, and the field as a whole has revealed how central humans'

relationship with them is to maintaining health and properly understanding and treating disease.

In order to define these host-microbe relationships with utmost precision, the field has taken a particular interest in the molecular interface between foreign bacterial cells and the mammalian immune system. At the broadest level, the eukaryotic host's first line of defense consists of physicochemical properties such as acidic stomach pH, secretory mucus, cationic antimicrobial peptides (AMPs), and peristaltic flow. Defense tier two, innate immunity, relies upon germline-encoded sensors of molecular patterns common to many microorganisms. Broad expression of these sensors across many different cell lineages in distantly-related animal taxa leave the host ready to respond within minutes at many different tissue sites. Finally, the third tier responses of adaptive immune cells in mammals invoke highly specialized recombined antigen receptors to develop exquisite specificity and immunological memory over several days and weeks. Virulent pathogens exhibit many molecular tricks by which they manipulate, inhibit, and subvert host immune responses in order to persist, thus provoking additional levels of activation in host immune cells. In contrast, most commensal bacteria that comprise the microbiota require little involvement from the host at all. Because the first and second barrier defenses are capable of keeping commensals out of the submucosal space, adaptive immunity is not typically invoked against the majority of commensals. In the cases in which commensal strains do induce adaptive immunity, the basic research is still underway to

discover what signals are engaging these innate and adaptive activation pathways. In short, many of the classical principles of immunology (e.g., those found in “Janeway’s”) were written based on evidence gleaned from pathogenic infections and not based on the study of commensals. This work humbly seeks to contribute its findings to the growing body of immune principles by which hosts respond appropriately to commensal microbes.

1.1.B. Endogenous IgA-coating of microbes in inflammatory bowel disease patients and healthy subjects

Mucosal immune responses have been examined for decades, as a surveillance and defense mechanism that maintains integrity of a key barrier site (McDermott et al., 1980; Tomasi et al., 1965; Underdown and Schiff, 1986). Studies of mucosal immunoglobulin (Ig) production have determined that secretory IgA (sIgA) is the most abundant Ig isotype produced in the human body, with estimates of daily output ranging from 0.006-2.2 g IgA/day (Meillet et al., 1987). Even though some baseline fraction of this humoral response is directed against food antigens, as evidenced by detectable levels of sIgA in germ-free (GF) mice, the vast majority of sIgA is produced in response to commensal gut bacteria (Hapfelmeier et al., 2010).

One longstanding paradigm of this mucosal sIgA response is known as “immune exclusion”, whereby host production of sIgA, among other mucins and AMPs,

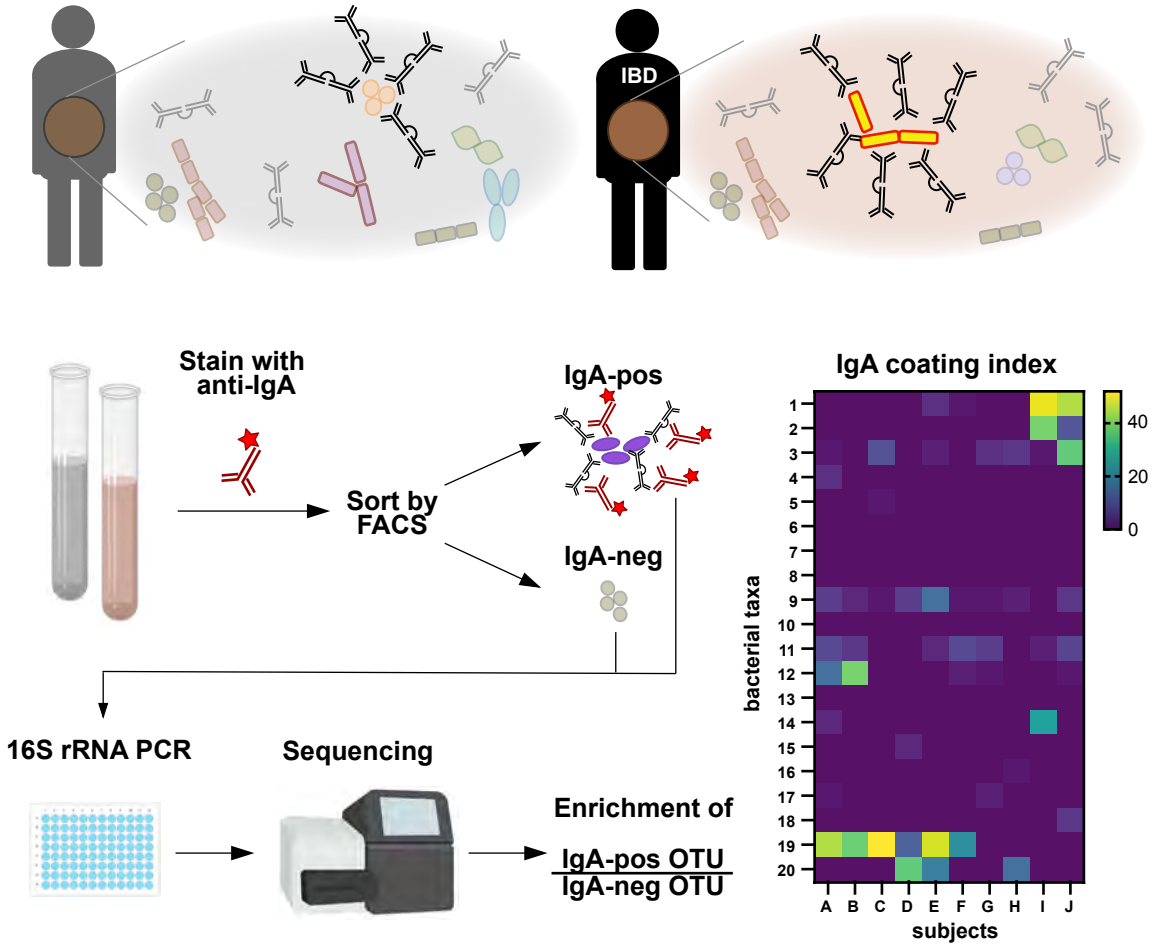

Figure 1. Evaluation of mucosal immune response to gut commensal bacteria by IgA-SEQ reveals previously unappreciated highly IgA-coated taxa. (A) Schematic illustrating the IgA-SEQ experimental approach, and an theoretical heatmap of IgA coating index (ICI) scores for 20 bacterial taxa across ten human subjects.

limits antigen access through the gut epithelium (Stokes et al., 1975; Williams and Gibbons, 1972). Later, after the development of more sophisticated imaging techniques, other groups discovered that this immune exclusion seems to maintain an appreciable separation between host epithelium and commensal microbes, akin to a “demilitarized zone” (Vaishnava et al., 2011). Although the variability of this separation has been debated and other models proposed (Bergstrom et al., 2020), many established experimental systems have shown that this sIgA response indeed defends against protein toxins, infectious bacterial pathogens, and even enteric viruses (Bouziat et al., 2017; Lycke and Holmgren, 1986; Moor et al., 2017).

Despite these studies, lingering questions remain concerning the mucosal immune response to commensal microbes. Are all commensal-derived antigens viewed with equal weight? Does the host response to a given taxonomy hold true across individual humans? To address these questions, Palm, de Zoete, and colleagues developed IgA-SEQ as a technology that combines fluorescence-activated or magnetic cell sorting (FACS or MACS, respectively), with high-throughput 16S rRNA gene sequencing to evaluate the endogenous IgA response to each taxon of commensal bacteria in each subject of interest (Figure 1) (Palm et al., 2014).

Using IgA-SEQ they demonstrated that healthy humans mount robust IgA responses to a variety of commensals, with certain organisms like *Akkermansia*

sps., Dorea sps., and *Ruminococcus sps.* being routinely IgA-coated at high magnitudes. Yet, there was also a clear dysregulation of IgA coating of commensals in inflammatory bowel disease (IBD) patients, with substantially higher mean levels of fecal bacteria IgA-coating in both Crohn's disease (CD) and ulcerative colitis (UC) compared to healthy controls. These findings clearly implicated the mucosal immune response to commensal gut bacteria in the etiology of human IBD, as well as provided the field with a high-throughput tool for assessing said responses from new angles.

Besides identification of new and noteworthy immunologically active taxa in the human gut, these data also reinforced the paradigm that certain commensal microbes might confer more risk on their hosts than others through an opportunistic strategy, which has been examined generally by other groups (Lozupone et al., 2012). The study of opportunistic pathogens traces its origins back to the mid-1960s, where bacterial isolates belonging to genus *Pseudomonas* were found to cause infection in goldfish only if accompanied by certain experimental insults (Bullock, 1965). Normal feeding of these fish with *Pseudomonas* did not result in infection, but only after removal of a few scales or direct injection of the bacteria would pathogenic infection ensue. Similarly, colonization of humans by *Pseudomonas* is not causative for the development of disease, as evidenced by the isolation of many *Pseudonomas* strains from healthy subjects. Rather, only in the context of CFTR mutations does *P. aeruginosa* lead to cystic fibrosis (Kubesch et al., 1993; Riordan et al., 1989).

The aforementioned studies of microbial IgA coating in the context of IBD have demonstrated a similar scenario in animal models: a subset of highly IgA-coated commensal bacteria seems to be causative for a mouse model of IBD under perturbed conditions only, whereas the mice colonized with the same microbes remain healthy under homeostatic conditions (Palm et al., 2014). Hence, we use the term “pathobiont” acknowledging the high degree of overlap with the term “opportunistic pathogen”, both of which describe a co-dependence on microbial factors and host factors.

1.1.C. Challenges in Culture-dependent approaches

The study by Palm et al. and this project both face the same challenge microbiologists have been facing for more than 150 years. The development of axenic culture methods seeks to mimic natural biochemical conditions in sterile broth formulations, such that microbes can be studied in pure isolation (Sullivan, 1905). The vast majority of environmental microorganisms remain fastidious and resistant to these attempts at *in vitro* culture, but advances in sequencing technology have enabled many environmental metagenomic studies that are growth-agnostic. Many challenges remain in culture methods for the capture of broadly diverse bacterial strains. While some laboratories make use of bioreactors to establish ongoing replenishment of culture conditions that simulate those of the gut, ours and many other laboratories rely on flexible-film anaerobic

culture chambers. Both the gaseous benchtop environment and all reagents and media are prepared without oxygen in order to preserve a semblance of the vertebrate gut environment. In this way, we can cultivate many human gut commensal anaerobes of interest and perform most standard microbiological manipulations under strict anaerobic conditions. Nonetheless, these conditions are still distant approximations of the gut environment itself, and largely fail to represent the effects of input and output streams, host-derived molecules, diet-derived complex polysaccharides, and biophysical flow forces on the astounding microbial ecology that exists *in vivo*. Rather than trying to recapitulate mammalian biology *in vitro*, our efforts largely seek to glean as much information as possible about host-microbe interactions by colonizing gnotobiotic mice defined bacterial consortia that differ by only one strain at a time, and analyzing primary samples from these mice.

1.2. Results

1.2.A. Isolation of human-derived *Allobaculum* species by culturomics

Using IgA-SEQ, we identified two human stool samples that contained highly IgA-coated commensals belonging to family *Erysipelotrichaceae* genus *Allobaculum*, each originating from an independent ulcerative colitis patient. We sought to isolate these *Allobaculum sps.* by the following method. Briefly, we plated suspensions of stool bacteria anaerobically on rich agar plates and

allowed colonies to grow for 48 hours. We picked hundreds of colonies into microplates and allowed liquid broth cultures to grow for 24-48 hours. These liquid cultures were then screened by high-throughput multiplexed 16S rRNA gene sequencing for identification of the bacteria growing in each well, followed by two more rounds of plating and colony picking and 16S screening until verification of pure *Allobaculum* isolates. Full length 16S rRNA gene sequences of these isolates are included in Table 1. Whole genome sequencing and hybrid assembly efforts also revealed that each strain carries a single chromosome of length 3.1 Mb. Average nucleotide identity (ANI) between these two genomes was found to be 0.80, indicative of two different species within genus *Allobaculum*. Along with our microbiologist colleagues at Universiteit Utrecht, we have recently established these strains as *Allobaculum mucolyticum* and *Allobaculum filumensis* (van Muijlwijk et al., 2021).

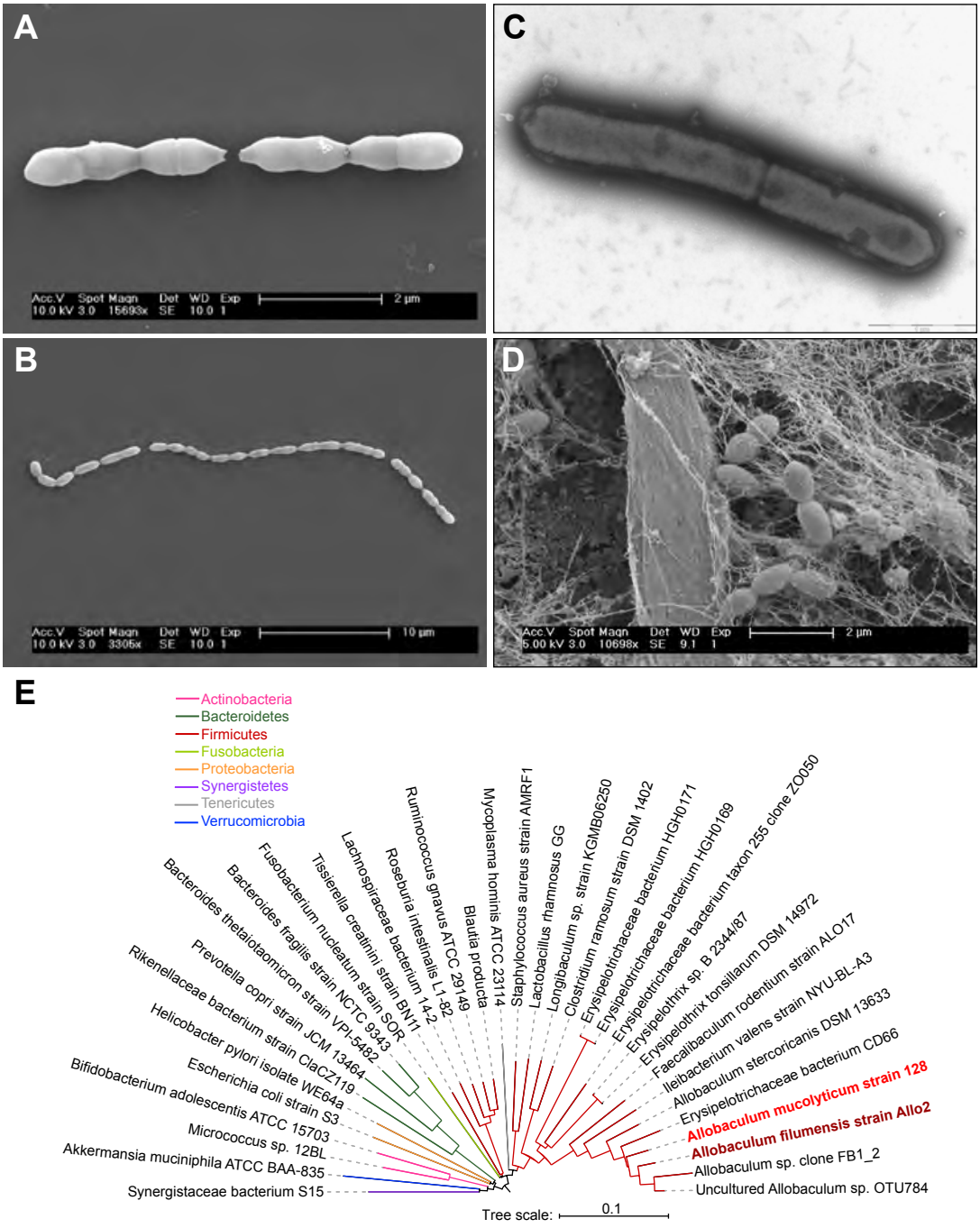

Figure 2. *A. mucolyticum* displays an enchained growth morphology *in vitro* and *in vivo* by electron microscopy (EM). (A-B) Scanning EM of *in vitro A. mucolyticum* broth cultures. Scale bar 2μm in (A), 10μm in (B). (C) Transmission EM of negative-stained *A. mucolyticum.* Scale bar 1μm. (D) SEM of WT murine colonic tissue mono-colonized with *A. mucolyticum*. Scale bar 2μm. (E) Phylogenetic tree constructed using 16S rRNA gene sequences from eight different bacterial phyla, including members of family Erysipelotrichaceae, using neighbor-joining method, bootstrapped to 1,000 replicates.

Table 1. Sequence identities of novel human-derived *Allobaculum sps.*

Isolate	**16S rRNA gene sequence**
128 (*A. mucolyticum*)	ATAACCTGCCCGTACCCGGGGGATACGCTTTGGAAACGAAGTCTAAAACCCCATAG GAAAGAAGAAGGCATCTTCTTCTTTTGAAACAAGCTTTTGCCTGGGGGACGGATGGA TCTGCGGTGCATTAGTTAGTTGGTGAGGCAAAAGCTCACCAAGACGATGATGCATAG CCGGCCTGAGAGGGCGAACGGCCACACTGGGACTGAGACACGGCCCAAACTTCTG CGGGAGGCAGCAGTAGGGAATTTTCGTCAATGGGCGCAAGCCTGAACGAGCAATGC CGCGTGAGTGAGGAAGGTCTTCGGATCGTAAAGCTCTGTTGCGGGGGAAAAAGGAA GCAGAAAGGAAATGGTCTGCTTTTGATGGTACCCCGCCAGAAAGTCACGGCTAACTA CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTATCCGGAATGATTGGGC GTAAAGGGTGCGCAGGCGGCGCGTCAAGTCTGAAGTAAAAGGTACAGGCTCAACCT GTGCAGGCTTTGGAAACTGGCGCGCTCGAGGACAGGAGAGGGCGGTGGAACTCCA TGTGTAGCGGTAAAATGCGTAGATATATGGAAGAACACCAGTTGCGAAGGCGGCCG CCTGGACTGTTACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAATAGGATTAGAT ACCCTAGTAGTCCACGCCCTAAACGATGAGGAGCAGGTGTCGGAGGGAGTACCCCG GTGCCGAAGCTAACGCAATGACTCCTCCGCCTGGGGAGTATGCACGCAAGTGTGAA ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGA AGCAACGCGAAGAACCTTACCAGGCCTTGACATCGGATGCGAAGACTCAGAGATGA GTTGGAGGCTATCATCCAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGGGA GATGTTCAGTTAAGTCTGGCAACGA
539 (*A. filumensis*)	GGCGCAAGCCTGAACGAGCAATGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAA ACTCTGTTGCGGGGGAAAAAGGAAGGGAAGAGGAAATGCTTTTCTTTTGATGGTACC CCGCCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC AAGCGTTATCCGGAATGATTGGGCGTAAAGGGTGCGCAGGCTGCGCGTCAAGTCTG AAGTGAAAGGTACGGGCTTAACCGGTACAGGCTTTGGAAACTGGCACGCTAGAGGA CAGGAGAGGGCGGTGGAACTCCATGTGTAGCGGTAAAATGCGTAGATATATGGAAG AACACCAGTTGCGAAGGCGACCGCCTGGACTGTTGCTGACGCTCAGGCACGAAAGC GTGGGGAGCAAATAGGATTAGATACCCTAGTAGTCCACGCCCTAAACGATGAGGAG CAGGTGTCGGAGGGAGGACCCCGGTGCCGAAGCTAACGCAGTGACTCCTCCGCCT GGGGAGTATGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG CGGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACA TAGGACGCGAAGACTTAGAGAAAAGTTGGAGGTTACCGTCCATACAGTGGTGCATG GTTGTCGTCAGCTC

In vitro characterization of these isolates revealed a weak growth phenotype where both captured isolates grew to a maximum density of 4*10^6 CFU/mL in rich anaerobic broth culture (Gifu anaerobic media). Furthermore, exposure of *A. mucolyticum* to a panel of antibiotic compounds revealed sensitivity to Ampicillin, Cefazolin, Ciprofloxacin (MIC ≥2 µg/mL), intermediate sensitivity to Metronidazole and Vancomycin (≥12 µg/mL), and resistance to Streptomycin (≥256 µg/mL).

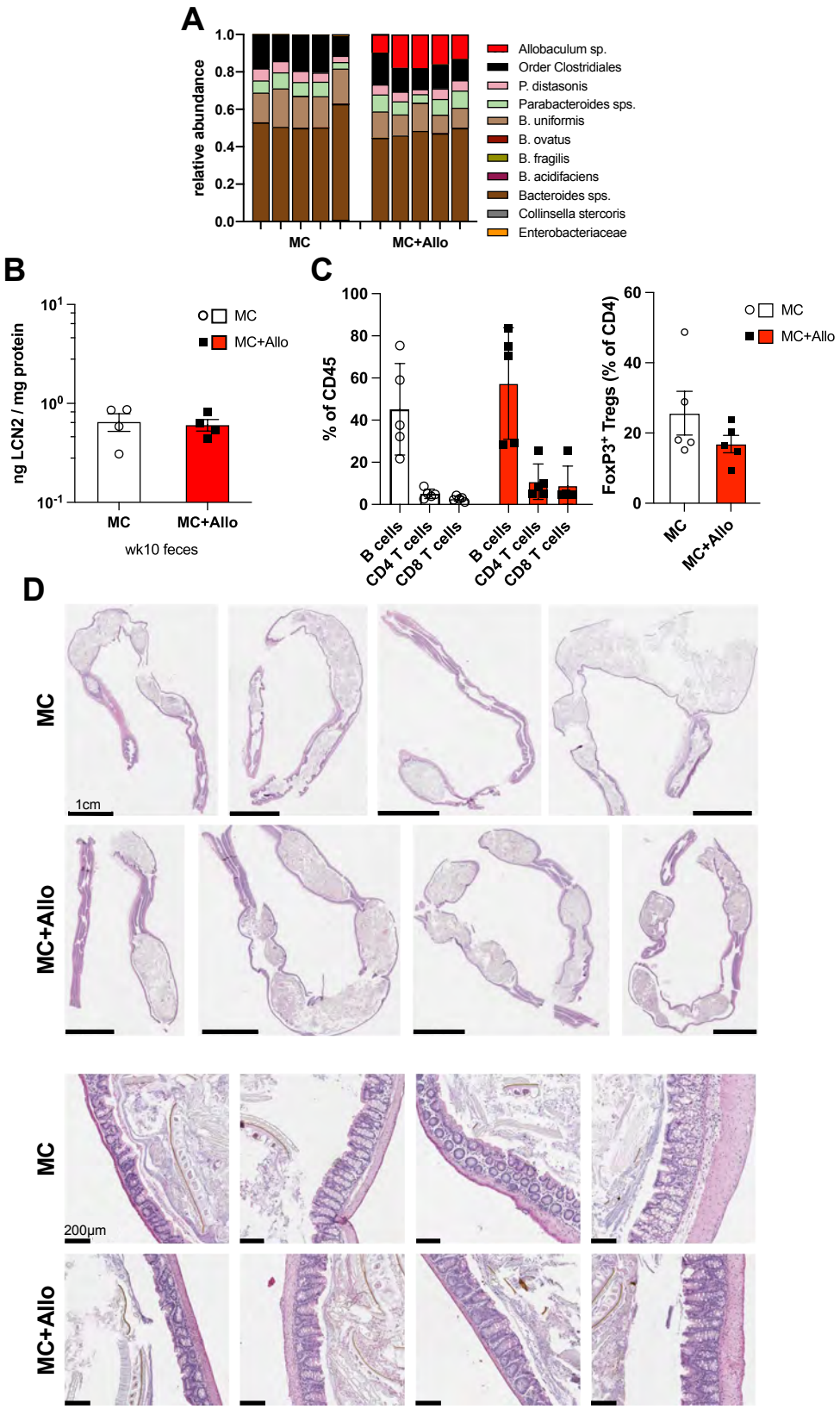

Figure 3. *A. mucolyticum* fails to induce spontaneous inflammation in WT mice at baseline. (A) Fecal microbiota profiling by 16S rRNA sequencing. (B) Fecal liocalin-2 (LCN2) at 10 weeks post-colonization. (C) Colon lamina propria immunophenotyping by FACS at 4 weeks post-colonization. (D) H&E-stained colon sections at 12 weeks post-colonization. Scale bars 1cm (top), 200µm (bottom).

Qualitatively, scanning electron micrographs of *A. mucolyticum* cultured *in vitro* revealed chains of dividing cells and morphology suggestive of cell-cell adhesive interactions (Figure 2A-B). Notably, we found no evidence of flagellin component gene homologs in the genome of *A. mucolyticum* nor gene homologs encoding components of pili or fimbriae. Confirming this bioinformatic analysis with soft-agar motility assays, neither isolate demonstrated a motile phenotype. Beyond these *in vitro* characterization experiments, we next sought to establish a mouse model of gut colonization by human commensal *Allobaculum sps.*

1.2.B. Colonization of gnotobiotic mice by *A. mucolyticum* does not result in detectable phenotypic changes

We introduced defined bacterial consortia into germ-free (GF) WT C57BL/6 mice by a single oral gavage and evaluated fecal microbiota by 16S rRNA gene sequencing to confirm colonization conditions in the guts of these mice (Figure 3A). Although we hypothesized that colonization with a non-inflammatory mock community (MC) plus *A. mucolyticum* would yield some amount of spontaneous immune activation compared to MC-colonized control mice, we observed no obvious phenotypic changes in the colons of these mice for up to 12 weeks post-colonization (Figure 3B-3D). First we monitored feces for the presence of occult blood, as well as levels of fecal lipocalin-2, neither of which revealed any significant inflammation (Figure 3B). Examining the colon tissue upon

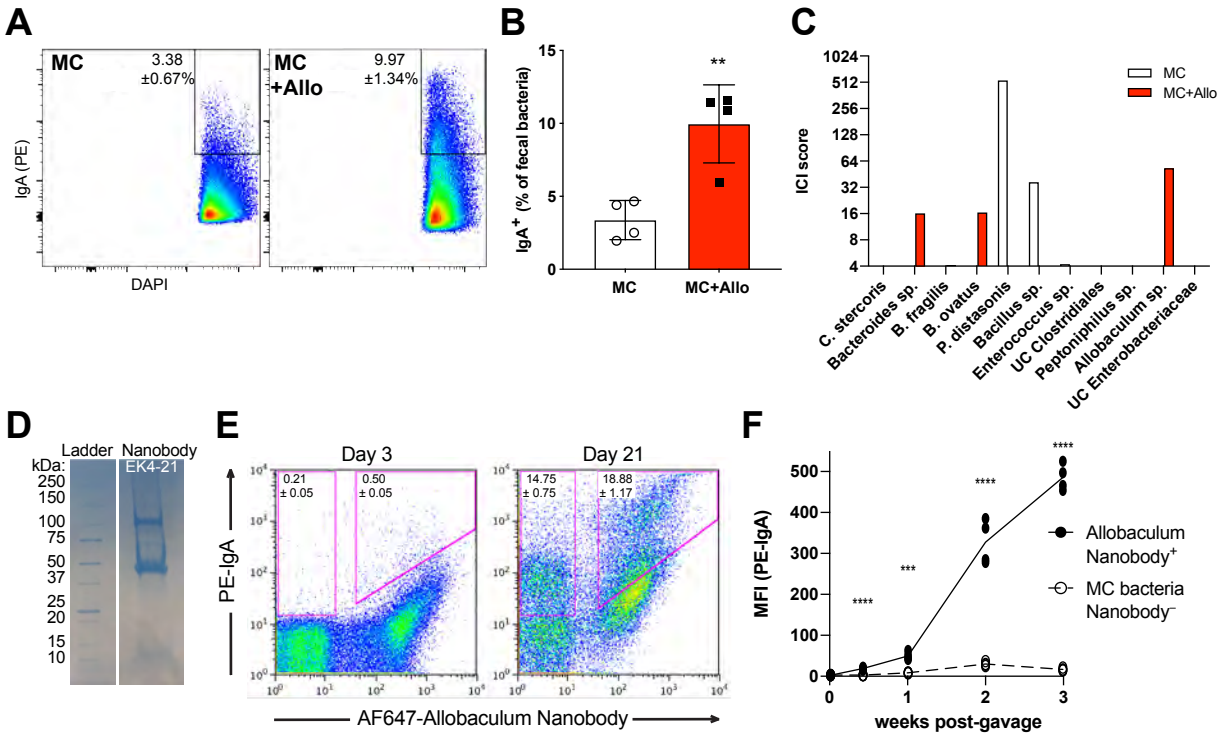

Figure 4. Development of an *A. mucolyticum*-specific nanobody allows for tracking of microbe-specific IgA response. (A-B) Representative FACS plots and quantification of fecal IgA coating from WT gnotobiotic mice colonized with MC or MC+*A. mucolyticum* at week 4 post-colonization. (C) IgA-coating index (ICI score) from IgA-SEQ analysis of fecal bacteria. (D) SDS-PAGE of recombinant nanobody raised against *A. mucolyticum*. (E-F) Representative FACS plots and quantification of *A. mucolyticum*-specific IgA coating over time using the nanobody shown in (D).

euthanasia, we saw no differences in bulk B and T cell populations, nor in histological changes at the tissue level (Figure 3C-3D). These data confirmed the sufficiency of WT immune mechanisms to contain and tolerate xenografted human gut commensals, yet left us puzzled as to how *A. mucolyticum* was affecting mucosal immune responses.

1.2.C. Colonization of gnotobiotic mice by *A. mucolyticum* results in induction of antigen-specific mucosal and systemic antibodies

Based on the original finding that this taxa was highly IgA-coated in human samples, we sought to evaluate bacterial IgA coating in gnotobiotic mice colonized with MC or MC+*A. mucolyticum* (Figure 4A-4C). In a preliminary IgA-SEQ experiment using fecal bacteria, we found that *A. mucolyticum* was the most highly IgA-coated organism among the MC+Allo microbiota. Although we observed an even higher magnitude IgA-coating index (ICI) for *P. distasonis* in the MC control mice, we note that this was abrogated upon addition of *A. mucolyticum*, likely due to the reduced microbial complexity in the MC microbiota compared to a complete human microbiota (Figure 4C).

Next, to examine the temporal dynamics of IgA-coating, we developed a novel reagent for specific tracking of *A. mucolyticum*. Using selection of an ultra-diverse library of nanobodies displayed on yeast, positively selecting for *A. mucolyticum*-binding yeast and negatively selecting against MC-binding yeast,

we iterated this selection process for 4 rounds, ultimately cloning several *A. mucolyticum*-reactive nanobodies and expressing them in mammalian cells (Table 2) (Figure 4D). Using this newly developed nanobody reagent in a multicolor flow cytometry panel, we examined longitudinal fecal samples and found a strong and specific induction of IgA against *A. mucolyticum* rather than against bacterial strains belonging to the mock community, even in samples from co-colonized mice (Figure 4E-4F). These data allowed us to conclude that *A. mucolyticum* possesses some unique property inducing specific and notable IgA response at the gut mucosa. We speculate that this process may be T-dependent owing to its weeks-long induction kinetics, although this remains to be formally demonstrated using cellular depletion strategies or genetic models of T cell deficiency.

Table 2. Recombinant nanobody reagents designed for specific detection of *A. mucolyticum.*

Nanobody Clone	**AA_sequence**
EK4-1	MEFGLSWVFLVALFRGVQSQVQLQESGGGLVQAGGSLRLSCAASGNIS RYQAMGWYRQAPGKEREFVATIADGASTYYADSVKGRFTISRDNAKNT VYLQMNSLKPEDTAVYYCAVVSKQGHRRKYYFEYWGQGTQVTVSSGG GGSGGGGSGGGGSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVV SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
EK4-21	MEFGLSWVFLVALFRGVQSQVQLQESAAACEAGGSLRLSCAASGNISN SYAMGWYRQAPGKERELVAAIGYGSSTYYADSVKGALPLAAKREKHRV SADEQPETGRYRGVLLRGLGRSWLLGPGHPGDVSSGGGGSGGGGSG GGGSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

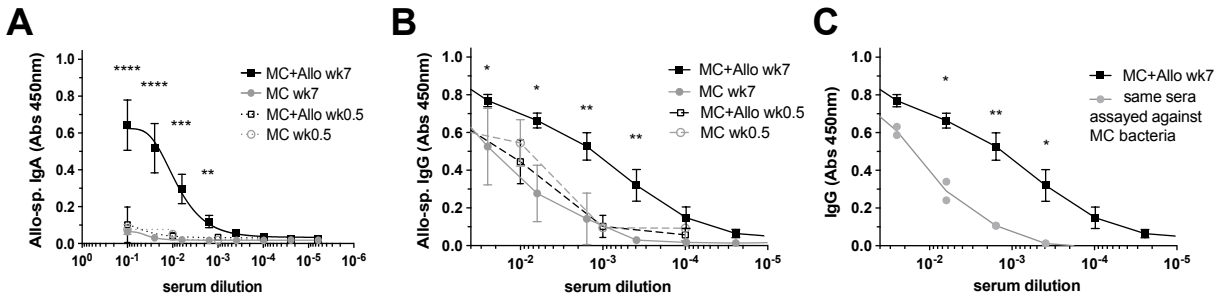

Figure 5. WT mice mount a systemic humoral response against A. mucolyticum at baseline. (A) A. mucolyticum-specific serum IgA ELISA. (B) A. mucolyticum-specific serum IgA ELISA. (C) MC bacteria-specific serum IgG ELISA. Nota bene: the solid black line is the same A. mucolyticum-specific IgG line shown in (B), for ease of comparison.

Beyond the mucosal antibody response, the literature provides only a few instances where gut commensal microbes induce concomitant serum antibody responses in mice (Ansaldo et al., 2019; Wilmore et al., 2018; Zeng et al., 2016). In humans, a few studies report bacterial-reactive IgG^+ intestinal plasma cells and bacterial-reactive serum IgG, yet disentangling this humoral immune response against bacteria from infection history and autoimmunity remains a murky challenge (Benckert et al., 2011; Fadlallah et al., 2019; Harmsen et al., 2012). This lack of clarity about the baseline humoral immune response to the commensal microbiota underscores a generally established paradigm of oral tolerance to diverse microbial and food antigens. These commensal bacteria, while conferring some degree of risk to hosts with immunodeficiencies, are largely worth tolerating because of the many benefits they provide their hosts.

To assess the degree of seroreactivity in our WT mice colonized with MC or MC plus *A. mucolyticum*, we analyzed serum binding to cultured bacteria by ELISA and found that, similar to fecal IgA, there was also a strong induction of *A. mucolyticum*-reactive serum IgA and IgG (Figure 5A-5B). Later subtyping showed that these *Allobaculum*-reactive antibodies were largely IgG1. We also confirmed that these antibodies were not reactive to the MC bacteria that co-colonize these animals, indicating that the response is not simply a wholesale production of “natural” polyreactive immunoglobulins (Figure 5C). Notably, we also found that this commensal-reactive serum IgG response held true in WT

mice colonized with MC+*A. filumensis* as well, indicating that there may be shared immunostimulatory features across different species of this genus (Table 3). These systemic antibody responses at steady state led us to conclude that *A. mucolyticum* is a notable immunostimulatory commensal strain that provokes the host immune system more so than other commensals, despite the absence of overt infection or cellular response in the colon lamina propria.

Table 3. Serum IgG titers against *A. mucolyticum* and *A. filumensis.*

Bacterial strain	**IgG titer, naïve**	**IgG titer, colonized 8 weeks**
A. mucolyticum	10^-1	8*10^-5
A. filumensis	10^-1	4*10^-4

1.2.D. *Allobaculum*-dependent susceptibility to murine colitis

Knowing that *A. mucolyticum* is notably immunostimulatory, we next sought to explore the effects of *A. mucolyticum* upon host inflammatory responses. To perturb the mucosal environment and initiate inflammation, we treated our mice *ad libitum* with dextran sodium sulfate (DSS) in drinking water. This widely-used animal model of acute ulcerative colitis disrupts the intestinal epithelial barrier and facilitates translocation of gut microbes into the mucosa where they provoke rapid inflammatory responses from the host. After treating WT gnotobiotic mice with 2% DSS for 7 days, we observed a dramatic increase in inflammatory pathology in animals colonized with MC+*A. mucolyticum* compared to those

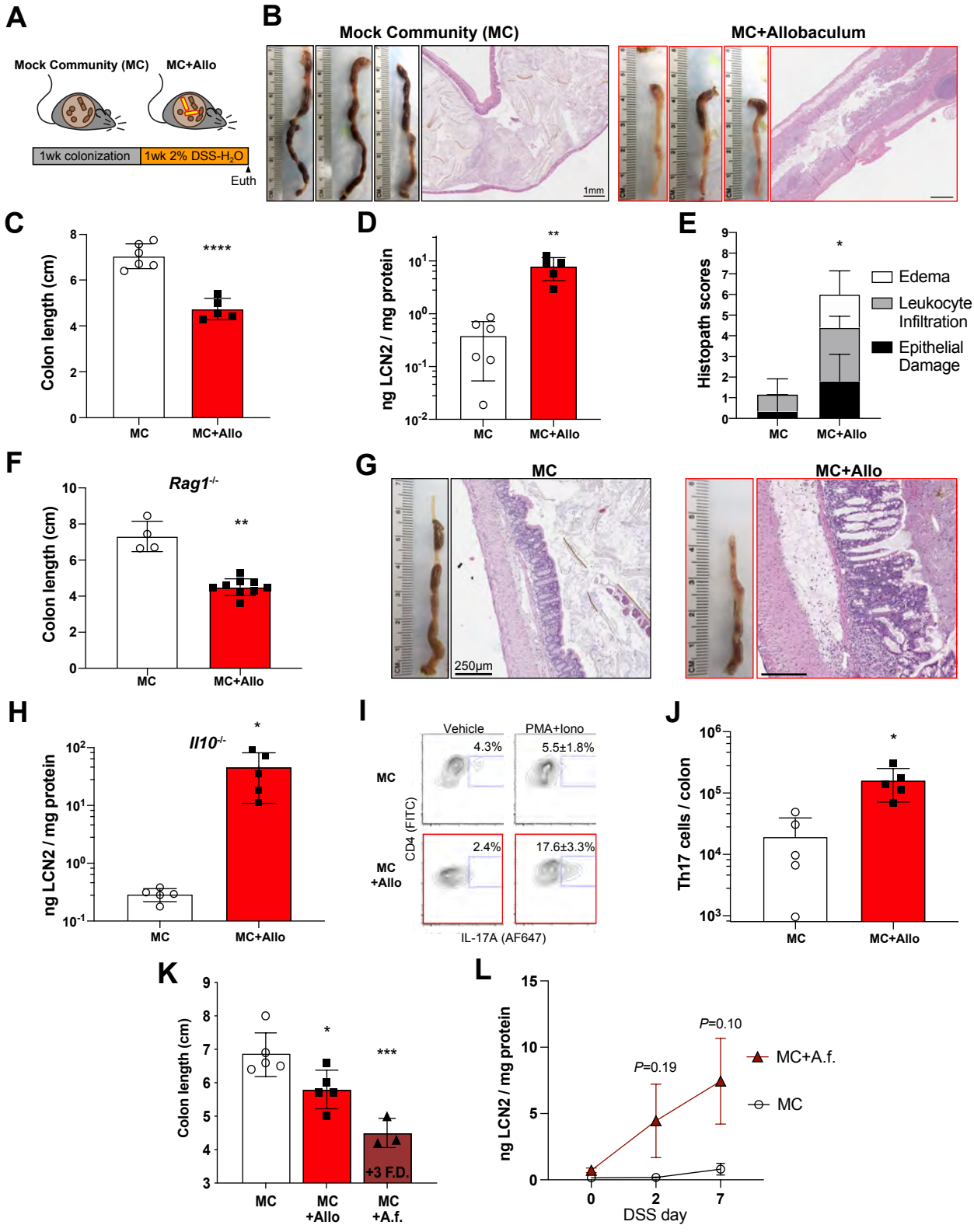

Figure 6. *A. mucolyticum* predisposes gnotobiotic mice to severe colitis in multiple models. (A) Experimental schematic. (B) Colon gross pathology and histology, (C) colon length, (D) fecal lipocalin (LCN2), and (E) histopath scores on DSS d7 in WT mice. (F-G) Colon length and colon histology in Rag1$^{-/-}$ gnotobiotic mice. (H-J) Fecal lipocalin, colon lamina propria CD4^{+} T cell cytokine staining, and quantification in Il10$^{-/-}$ gnotobiotic mice. (K-L) Colon length and fecal lipocalin in WT gnotobiotic mice colonized with *Allobaculum filumensis*.

colonized with MC alone (Figure 6A-6E). A pronounced influx of leukocytes, ulceration of the epithelium, and shortening/thickening of the colon characterized this *A. mucolyticum*-dependent response. This effect also held true in immunodeficient *Rag1*$^{-/-}$ mice colonized and treated with the same conditions, indicating that this *A. mucolyticum*-dependent colitis phenotype does not depend on adaptive immunity (Figure 6F-6G). Similarly, we also found that DSS colitis was exacerbated in WT mice colonized with MC+*A. filumensis* compared to MC alone (Figure 6K-6L). We speculate that these two species within genus *Allobaculum* share some common molecular feature or colonization strategy leading to this severe inflammation.

In an effort to represent certain features of human IBD etiology more accurately (gradual disease onset, involvement of autoimmune lymphocytes, absence of chemical agents), we also examined colitis in a spontaneous mouse model. Colonizing gnotobiotic *Il10*$^{-/-}$ mice with MC+*A. mucolyticum* resulted in a colitis progression that typically manifested at 6-8 weeks post-colonization whereas MC-colonized mice remained healthy at that time (Figure 6H-6J). These data lead us to conclude that *A. mucolyticum* is indeed a pathobiont that initiates a host response against otherwise-tolerated commensal bacteria, and in the right inflammatory milieu or loss of tolerance drives aberrant immunopathology. These data are in keeping with many human studies that show carriage of particular opportunistic pathogens by healthy human subjects. Only under abnormal circumstances, like genetic or environmental insults, do these microorganisms

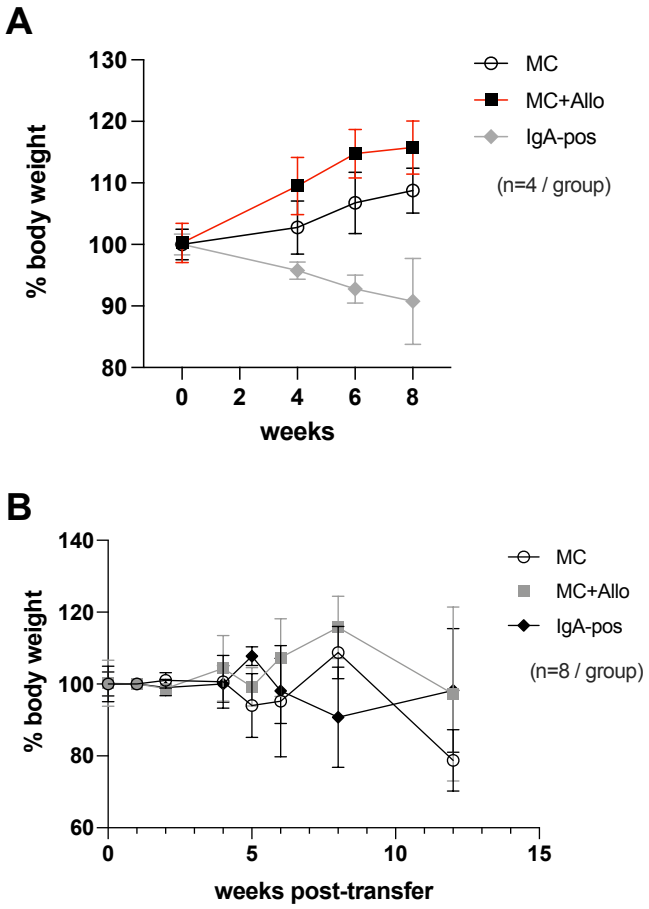

Figure 7. *A. mucolyticum* fails to exacerbate naïve T cell transfer colitis. CD45RB^{hi} splenic T cells were FACS sorted from SPF donor mice and adoptively transferred into irradiated Rag1$^{-/-}$ recipient gnotobiotic mice colonized with defined microbiota. Mice were monitored for body weight change over time as well as any signs of intestinal inflammation. (A) Experiment #1, (B) Experiment #2.

lead to major health problems, but they nonetheless merit study and characterization for deeper understanding of host responses to the gut microbiota both at steady state and during inflammation.

To model the many cases of IBD in humans that exhibit a signature of autoimmune T cell involvement, we also examined the contribution of *A. mucolyticum* to T cell-driven colitis. Naïve CD45RB^{hi} T cells were FACS-sorted from spleens of donor mice, to the exclusion of regulatory T cells, and adoptively transferred into recipient Rag1$^{-/-}$ gnotobiotic colonized with MC+*A. mucolyticum* or MC alone. In our hands these mice failed to develop colitis in a consistent microbiota-driven manner, instead exhibiting stochastic effects of inflammation across three different microbiota groups (Figure 7). We take these data to indicate that there is unlikely to be a strong induction of *Allobaculum*-specific pathogenic effector T cells in our immune-replete murine colitis models (whether WT or *Il10*$^{-/-}$), but does not exclude the possibility of human T cell involvement in *Allobaculum*-colonized IBD patients.

1.2.E. *A. mucolyticum* inhabits the midgut niche and penetrates into crypts

Based on a body of work that posits the biogeographical location of gut commensals as an important factor in the development of host inflammation, we hypothesized that proximity to the host epithelium might be a factor underlying *A. mucolyticum*-dependent antibody responses and risk of colitis. To test this

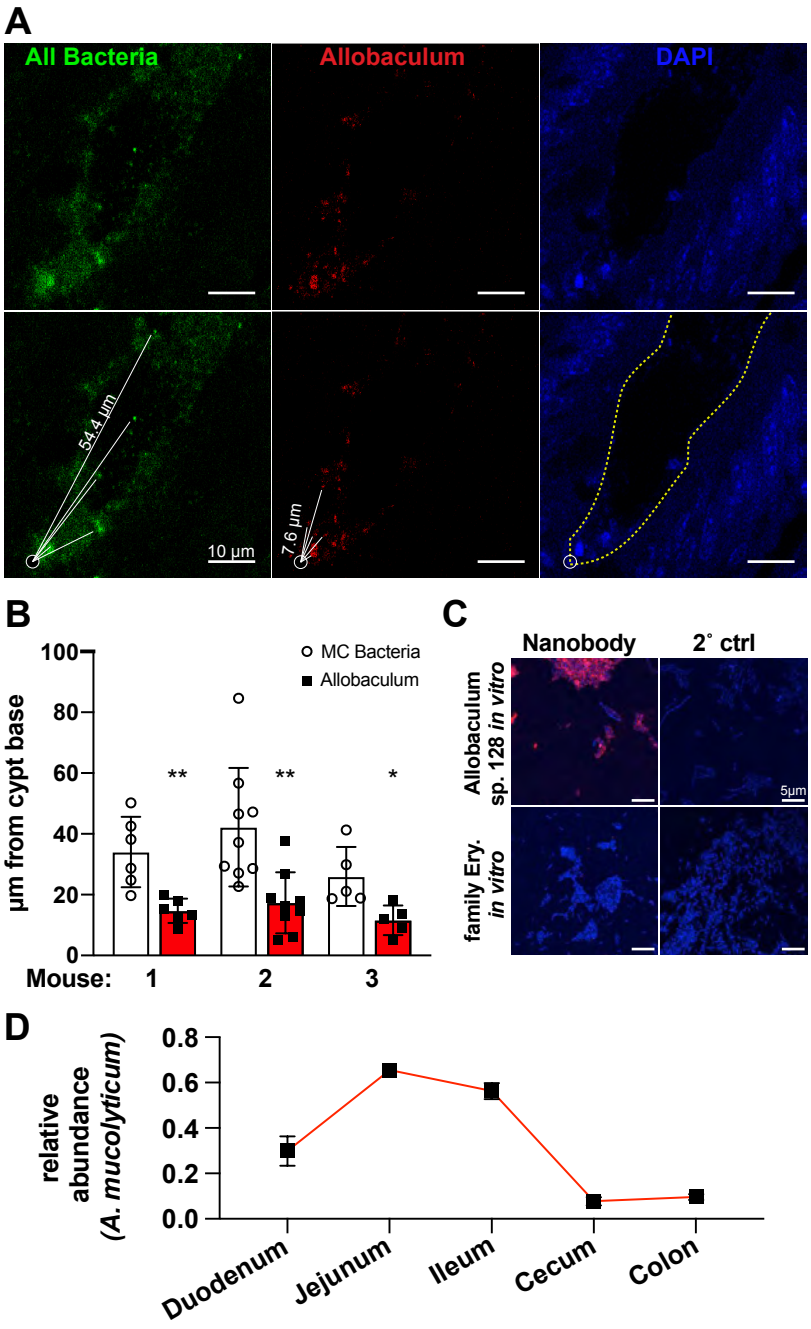

Figure 8. *A mucolyticum* inhabits ileal crypts to a greater extent than other control bacteria. (A) Representative confocal micrographs of ileal sections of WT gnotobiotic mice colonized for 4 weeks with MC bacteria + *A. mucolyticum*. From left to right, pan-bacterial stain in green, *A. mucolyticum*-specific nanobody in red, and nuclei (DAPI) in blue. Scale bars, 10µm. (B) Quantification of average bacterial distance from crypt in n=5-8 crypts per mouse, across three mice. (C) Control stains demonstrating *A. mucolyticum* (sp. 128) binding by nanobody *in vitro*. Scale bars, 5µm. (D) *A. mucolyticum* relative abundance in various gut segments.

hypothesis we examined gut crypts for the presence of MC bacteria and *A. mucolyticum* by confocal microscopy, partitioning our efforts into examining the colon and the terminal ileum. Using the same *A. mucolyticum*-specific nanobody reagent as above, we were able to visualize *A. mucolyticum* cells *in situ* as well as control bacteria of other taxa. We found that *A. mucolyticum* cells were more likely to be found nearer to the base of ileal crypts than control MC bacteria, but this was not true in the colon (Figure 8A-8C). Furthermore, we found the relative abundance of *A. mucolyticum* to be substantially higher in the lumen of the ileum than that of the colon (Figure 8D). These findings suggest that biogeographical separation from host epithelial cells may represent a physicochemical defense mechanism that *A. mucolyticum* perturbs more so than other commensal bacteria, leading to greater immune responses at baseline and exacerbated immunopathology after inflammation is underway.

1.3. Conclusions

From these data we conclude that *Allobaculum mucolyticum* and *Allobaculum filumensis* are two novel immunogenic isolates derived from human ulcerative colitis patients that merit further investigation. These commensal bacteria may contribute to the etiology of inflammatory bowel diseases, but are also broadly useful for their ability to reveal the rules of engagement between the mucosal immune system and human microbiota. Induction of antigen-specific mucosal and systemic antibodies in the absence of overt histological changes to the

colonic tissue demonstrate surveillance of the intestinal compartment by immune cells and their ability to respond when the need arises. For this reason, we contend that species of the genus *Allobaculum* are notable and unusual members of the human microbiota in this regard.

In light of the genetic and environmental insults that likely precipitate inflammatory bowel disease, we conclude that *Allobaculum sps.* are highly notable candidates for their ability to drive severe colitis in gnotobiotic mice colonized with defined bacterial consortia. Perhaps because of its ability to thrive in the midgut niche and penetrate deep into ileum crypt niches more so than other commensals, *A. mucolyticum* results in stark phenotypic changes in both the intestinal immune compartment and the systemic humoral response.

1.4. Pitfalls, weaknesses, & alternative interpretations

A widespread pitfall of the microbiome field is seen in the range of studies that occupy the extreme opposite ends of the microbial community spectrum: overly simplified and vastly complex. Mechanistic conclusions are routinely drawn from experiments using mono-colonized animal models or inferences drawn from analyses of human stool samples. This study seeks to find some middle ground between the two by using a defined consortium of bacterial strains that are relevant to the human microbiota and approximate some of its many metabolic and ecological functions. Yet, because these strains only represent three major

phyla of the human microbiota, one could rightly argue that these experiments only represent a small fraction of the complex microbial ecology found in the human gut. This weakness means that there are potential axes of microbe-mediate activation of host immunity that are possibly being missed, even those that might affect induction of IgA-coating of *Allobaculum* or the broader *Allobaculum*-induced inflammatory response.

We have yet to undertake detailed characterizations of intestinal epithelial cells (IECs) in vivo that might reveal the earliest activation signals triggered by *A. mucolyticum.* Experiments with immortalized epithelial cell cultures *in vitro* demonstrated no obvious cytotoxicity or cell death in co-culture with *A. mucolyticum*, but our *in vivo* data certainly lack formal examination of IECs. In theory, *A. mucolyticum* likely activates innate immunity in IECs within the first few hours and days of colonization. Whether these activation steps influence one particular IEC lineage or a broader signaling pathway remains to be seen, but could provide important clues as to the host-microbe molecular interactions that dictate downstream phenotypic outcomes in mice.

Similarly, we have not fully characterized the extent of *A. mucolyticum* colonization or activation of nearby inductive sites, such as Peyers patches (PP), isolated lymphoid follicles (ILF), and draining mesenteric lymph nodes (MLN). We speculate that due to its stark presence in the ileum, *A. mucolyticum* may profoundly impact these lymphoid tissues that later give rise to the antigen-

specific Ig we have detected in feces and serum. This is in keeping with the global absorptive functions of the small intestine, allowing passage of nutrients and other antigens through the epithelium, and in contrast to the colonic epithelial barrier, which is much tighter and more immunologically “ignorant” overall. Thus, we are performing ongoing experiments to parse out the absolute quantity of various colonizing strains in all niches of interest, in order to precisely map where the greatest antigenic load exists *in vivo*, and to help guide our understanding of this unique immunostimulatory commensal and its interactions with the host.

There is ongoing debate in the IgA biology field about the dominant cellular contributors to mucosal antibody responses: B cells alone producing T-independent IgA, or T-dependent IgA (Bunker et al., 2017; Bunker et al., 2015; Hirota et al., 2013; Lindner et al., 2012; Macpherson et al., 2000). Of course this debate is greatly complicated by discrepancies in murine studies versus human studies, but the central issue generally boils down to varying emphases on global patterns of IgA binding across entire microbiota versus targeting of IgA to particular taxa in an antigen-specific manner. In our models we have yet to dissect the cellular basis of *Allobaculum*-specific IgA production, whether it depends strictly on the presence of murine T cells or not. This knowledge would perhaps clarify certain aspects of this unique host-microbe relationship, such as generation of long-lived memory responses specific for this commensal, as well as the implication of effector T cells in chronic immunopathology.

1.5. Potential solutions and future directions

Ongoing experiments seek to examine the ability of *A. mucolyticum* to induce inflammation via intestinal epithelial cell activation, as well as the downstream steps leading to immunity in antigen presenting cells and adaptive immunity. We hope these pathways will provide clues as to what type of bioactivity or molecular signal *A. mucolyticum* is exerting upon hosts in settings of close biogeographical proximity, such as in the ileum crypts. We also seek to understand how these signals are relayed to antigen-specific lymphocytes, and in what lymphoid tissues. Using various cellular depletion strategies and genetic backgrounds, we hope to soon determine the involvement of murine T cells in this anti-commensal antibody response and identify the location of this T cell priming, if it is detectable.

In order to address the challenges of a minimal defined bacterial consortium, one strategy we seek to leverage is natural microbial biodiversity. By capturing different strains and species within a genus—in this study, multiple isolates of genus *Allobaculum*—we can then compare the activities and genomes and proteomes of these isolates to determine which molecular components are unique or shared, thus narrowing in on candidate bioactive antigens. Both comparative genomics and epitope predictions *in silico* have begun to guide us towards the molecular basis of activation found in the *Allobaculum* genomes, but further screening of these candidate antigens is necessary and forthcoming (see

Chapter 3). This approach also helps us understand the complex ecology of the human gut, as swapping out different bacterial strains sometimes has profound phenotypic effects, and other times remains robust to these changes. We hope to build increasing complexity in our animal models, such that they more and more recapitulate the fullness of a human microbiota (Cheng et al., 2021). In this way we intend to begin decoding microbiota functionality that is both mechanistic and broadly relevant to the human gut.

Chapter 2. Epistatic interaction between divergent gut microbes reprograms host immune response

2.1. Introduction

2.1.A. Microbial community context leads to phenotypic variation between individuals

The study of bacterial pathogenesis has occupied microbiologists and immunologists for centuries, from Bubonic plague to Legionnaire's disease to Anthrax. Certain virulent bacterial pathogens have been identified and kept at bay through international vaccination campaigns and public health efforts (Girard et al., 2006; Jones, 2005; Organization, 2006). Yet, defense against the staggering diversity and ubiquity of bacteria remains a global challenge both for bona fide pathogens and opportunistic pathogens. For example, *Clostridium difficile* and *Staphylococcus aureus* are detectable in the gut and on the skin of healthy humans, respectively, without causing any spontaneous adverse consequences or infections. This is largely due to commensal microbial communities that normally provide a measure of colonization resistance render these opportunistic pathogens harmless (Kato et al., 2001; Nakatsuji et al., 2017; Niyogi et al., 1997; Tomczak et al., 2019). Yet, when the microbiota is perturbed, resulting in a "dysbiotic" state, a loss of colonization resistance often leads to *C. difficile* taking over the open niche and causing immunopathology (Vincent et al.,

2016). Likewise, *S. aureus* has been implicated in a variety of dire skin pathologies and intractable infections (Naimi et al., 2003).

A longstanding unresolved issue in the field stems from the fact that many opportunistic pathogen-colonized patients that remain healthy exhibit unknown correlates of protection. Stated another way, there is an incomplete penetrance of microbial effects upon disease. This issue can be seen clinically when a subset of patients colonized with *C. difficile* end up with life-threatening enteropathy while other colonized subjects remain completely healthy (Buffie et al., 2015; van Nood et al., 2013; Vincent et al., 2016; Weingarden et al., 2014).

Outside the realm of clinical infectious disease, there are other instances in which this incomplete microbial penetrance has been observed in animal models. Immunostimulatory commensal gut microbes have been examined extensively in mice, leading to the characterization of several model symbionts. Despite the use of inbred strains, mice, like their human counterparts, also exhibit variation in their individual immune responses to colonizing microbes. Segmented filamentous bacteria leads to induction of IL-17-producing T helper (Th17) cells in the gut, but this depends on the facility in which the mice are housed and the endemic microbiota therein (Atarashi et al., 2015; Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Lee et al., 2011; Wu et al., 2010). Human-derived *Bacteroides fragilis*, when engrafted into gnotobiotic mice, can colonize the colonic crypts, but only if that crypt is unoccupied at the time–it will be rejected by

other commensals that are prior crypt inhabitants (Lee et al., 2013). *Akkermansia muciniphila* in mice leads to induction of antigen-specific serum IgG, via follicular T helper (Tfh) cells, but with wide variation in titer that is microbiota-dependent (Ansaldo et al., 2019). These phenomena are widely encountered across institutions and labs, but studies that formally address this microbe-centric issue are lacking. Hence, this work seeks to address specific strain-level microbial interactions *in vivo* and document their effects upon host immune phenotype.

2.1.B. Epistasis in the gut microbiome: borrowing from genetics

In genetic regulatory networks, the interaction of multiple alleles and their aggregate result on phenotype is termed epistasis (Phillips, 2008). We invoke this concept in our microbiome study to describe the effects of a given microbe on an isolated host system suddenly changing when other microbial players are included in the community, just as other genetic alleles alter the effects of one allele (Lengfelder et al., 2019).

A considerable amount of work has been done to define pathways of intestinal carbohydrate metabolism that are dependent on multiple microbial taxa (Fischbach and Sonnenburg, 2011; McNulty et al., 2013; Pudlo et al., 2015; Sonnenburg et al., 2010; Sonnenburg et al., 2005; Tuncil et al., 2017). These pathways are often dependent on the activities of multiple cross-feeding strains and their polysaccharide utilization loci (PULs), which naturally fluctuate *in vivo*.

These examples of microbial epistasis in the context of gut metabolism are fairly clear, while epistatic effects upon immunological activation have yet to be elucidated. In addition to the response programs that are mounted solely by the host, our work seeks to define the specific microbe-microbe interactions that have profound consequences for reprogramming host immunity.

2.2. Results

2.2.A. Human microbiota discovery screen reveals *Akkermansia muciniphila* to be negatively correlated with *Allobaculum mucolyticum*.

Our studies of colitis in gnotobiotic mice suggested that immunostimulatory *Allobaculum* strains may play potentially causal roles in IBD. However, we also detected related *Allobaculum* strains in a meta-analysis of microbiome data from ostensibly healthy humans (American Gut Project) (McDonald et al., 2018). One potential explanation for this observation is that specific microbial taxa present in healthy humans may protect against the colitogenic effects of *Allobaculum.* To begin to examine this hypothesis, we established a human microbiota-associated gnotobiotic mouse-based screen to reveal potential relationships between *A. mucolyticum* and diverse bacterial taxa from the human gut microbiota. Briefly, we mono-colonized individually housed germ-free mice with *A. mucolyticum* for 24 hours before gavaging each mono-colonized mouse with one of 19 different healthy human stool samples. After seven days, we evaluated microbial

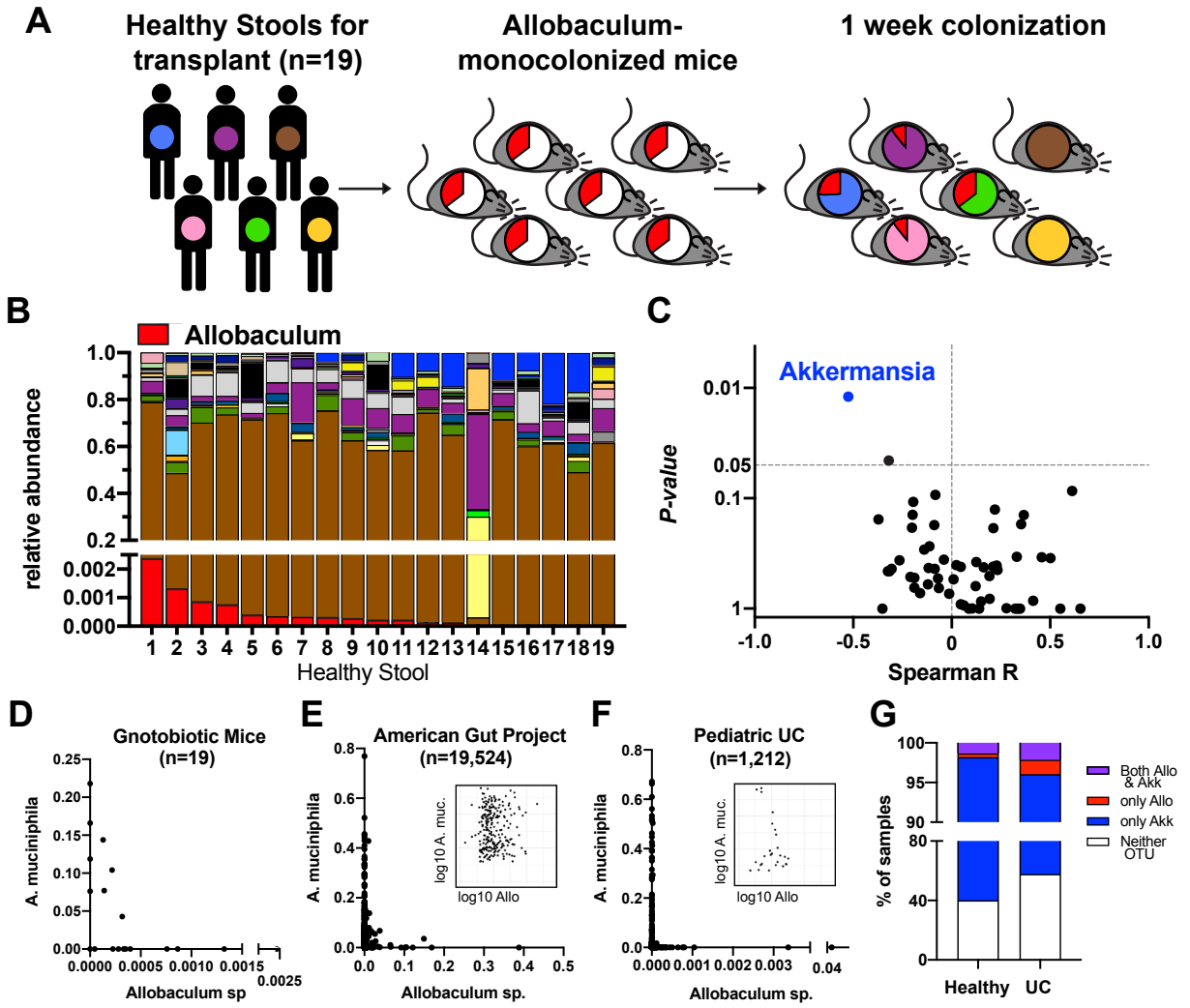

Figure 9. *A. mucolyticum* is inversely correlated with Akkermansia muciniphila in human microbiota-associated gnotobiotic mice. (A) Experimental workflow. (B) GF WT mice were gavaged with live *A. mucolyticum* culture, then with suspension of healthy human stool 24h later. Day 7 fecal pellets were collected for microbiota profiling (n=19). Bacterial OTU legend shown in Figure S4B. (C) Each genus-level OTU was tested for Spearman correlation to *A. mucolyticum* abundance. *A. mucolyticum* abundance was binary-transformed and logistic regressions were also fit for each OTU pair, allowing for calculation of log-likelihood ratios and P-values. (D) XY plot of data shown in (B). (E-F) Meta-analysis of human microbiome datasets: American Gut Project (McDonald, et al. 2018; QIITA study IDs: 48742, 51570, 52698, 53379, & 54454) and pediatric ulcerative colitis (UC)(Schirmer, et al. 2018).

community composition in all mice via 16S rRNA gene sequencing (Figure 9A-B). As expected, mice colonized with different stool samples harbored distinct microbial communities. Furthermore, we observed a range of *A. mucolyticum* colonization levels across these 19 unique community contexts (Figure 9B). This variation in *A. mucolyticum* abundance was not due to variation in overall microbial diversity as there were no significant differences in richness or evenness between samples containing *A. mucolyticum* and those lacking *A. mucolyticum* (Figure S1A). Thus, we hypothesized that specific microbial taxa may impact *A. mucolyticum* carriage or abundance. To identify such taxa, we calculated Spearman correlation coefficients for all genus-level OTUs paired with *A. mucolyticum* abundance and tabulated log likelihood ratios for each pairing. Remarkably, the well-known immunogenic mucinophile *Akkermansia muciniphila* (OTU_363731) exhibited the lowest Spearman coefficient (R = -0.52) and the most significant likelihood ratio (Figure 9B-D). To test whether this relationship between *A. mucolyticum* and *A. muciniphila* is generalizable to humans with naturally acquired microbiota, we assessed the relative abundance of these two taxa in publicly available large-cohort studies of healthy human volunteers (n = 19,524) and pediatric UC patients (n = 1,212) (McDonald et al., 2018; Schirmer et al., 2018). We found that *A. mucolyticum* and *A. muciniphila* exhibited a broadly similar anticorrelation to what we observed in our human-microbiota associated gnotobiotic mice (Figure 9E-F). Overall, these data reveal an inverse relationship between two phylogenetically distinct immunostimulatory commensal

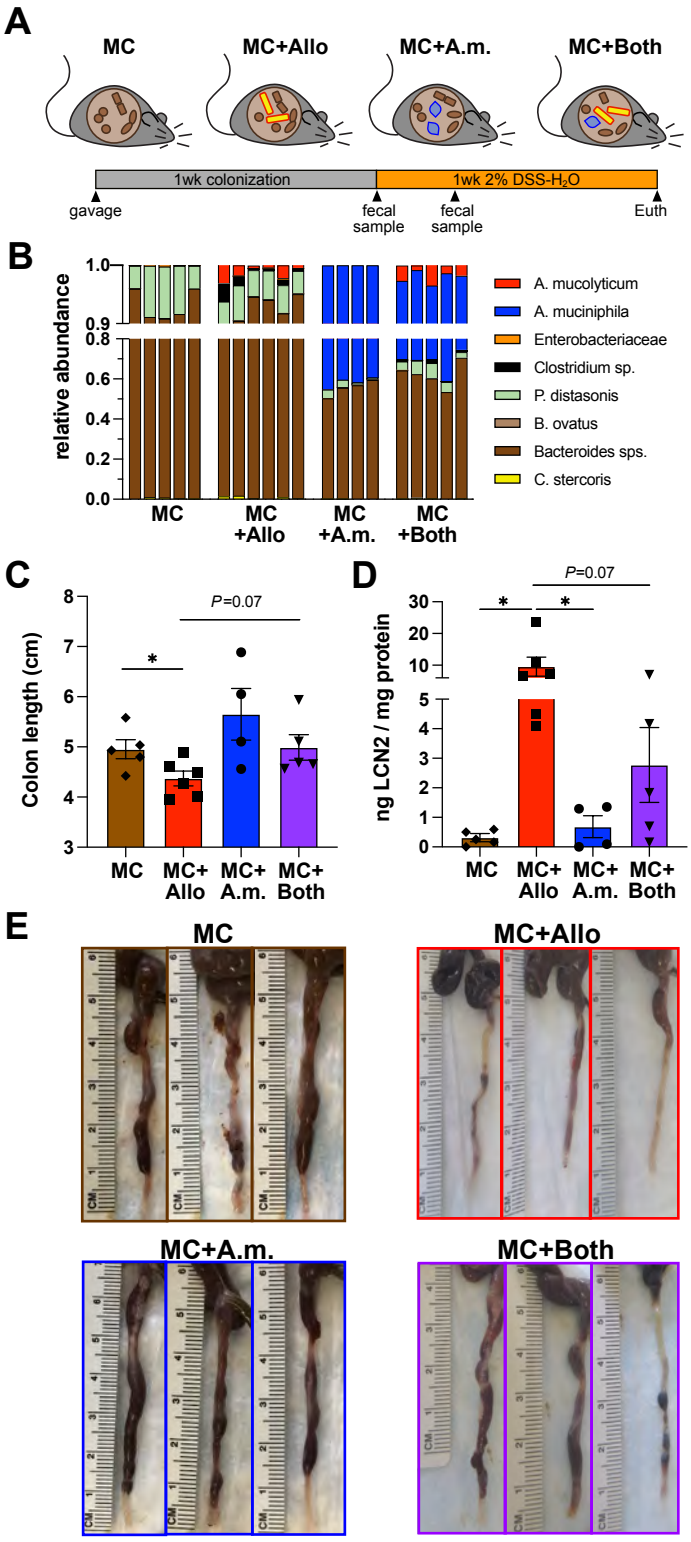

Figure 10. Co-colonization with *A. muciniphila* ameliorates *A. mucolyticum*-mediated colitis. (A) Experimental schematic. GF WT mice were gavaged with MC+*A. mucolyticum*, MC+*A. muciniphila* (ATCC BAA-835), or MC+Both *A. mucolyticum* and *A. muciniphila* and allowed to equilibrate for 1 week before initiation of DSS colitis. (B) Fecal pellets were collected, gDNA extracted, and bacteria profiled by 16S rRNA V4 amplicon sequencing (n=4-6 mice per group). (C-E) From wk1-2, mice were given 2% DSS-H2O ad libitum to induce acute colitis, which was assessed by colon length and fecal lipocalin-2 (LCN2) at euthanasia. Error bars show mean +/- SEM. (E) Gross colon pathology at euthanasia. Welch's t-test was used to compare each cell lineage across MC+A.m. vs MC+Allo groups; * P<0.05.

taxa and raise the possibility that *A. muciniphila* may influence *Allobaculum*-induced immune responses.

2.2.B. Pairwise effects of *A. mucolyticum* and *A.muciniphila* on colonic inflammation

To test the potential effects of *A. muciniphila* on *A. mucolyticum*-induced colitis, we colonized groups of WT gnotobiotic mice with either *A. mucolyticum*, *A. muciniphila* (type strain ATCC BAA-835), or both *A. mucolyticum* and *A. muciniphila* in the MC background and treated them with DSS (Figure 10A). Importantly, *A. muciniphila* and *A. mucolyticum* durably co-colonized mice in the context of this defined mock community, enabling us to examine the impacts of both taxa on immunity concurrently (Figure 10B). As expected, *A. mucolyticum*-colonized mice exhibited severe colitis after DSS treatment, as measured by fecal lipocalin and gross colon pathology. However, both *A. muciniphila*- and co-colonized mice displayed significantly lower levels of intestinal inflammation (Figure 10C-E). We also observed similar results using another strain of *A. muciniphila* that we captured by culturomics (in-house human isolate 2G4) (Figure S2). Notably, the effects of co-colonization on fecal *A. mucolyticum* abundance varied between experiments–in some cases co-colonization reduced overall *A. mucolyticum* abundance, while in others it remained largely unaffected. Even so, co-colonization still ameliorated colitis in mice where fecal *A. mucolyticum* abundance was similar between mice colonized with MC+*A.*

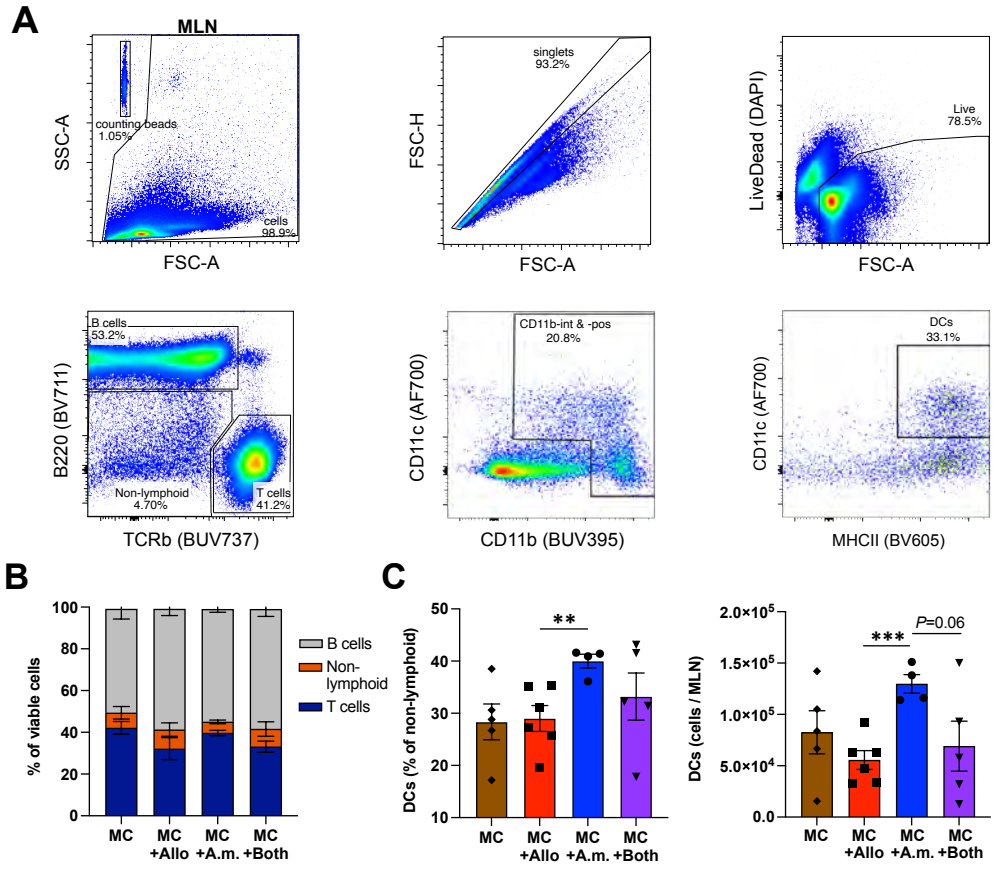

Figure 11. *A. mucolyticum* blunts *A. muciniphila*-induced DC accumulation in MLN. (A) Representative gating strategy for analysis of MLN cells performed in FlowJo (v10) after ≥ 100,000 events per sample were collected on a BD LSRII cytometer. (B) Immunophenotyping of MLN cell populations (% of viable cells), n=4-6 mice/group. (C) Quantification of DCs (Live B220$^-$TCRb$^-$CD11b$^+$CD11c$^+$MHCII$^+$). Welch's t-test was used to compare each cell lineage across microbiota groups; * P<0.05, ** P<0.01, *** P<0.001.

mucolyticum alone or co-colonized with *A. mucolyticum* and *A. muciniphila* (Figure 10B vs. Figure S2B). These data collectively demonstrate that *A. muciniphila* ameliorates pathological intestinal immune responses incited by *A. mucolyticum* in this acute model.

2.2.C. Pairwise effects of *A. mucolyticum* and *A.muciniphila* on antigen presenting cells

In addition to testing the effects of co-colonization on local intestinal inflammation, we also performed immunophenotyping of the mesenteric lymph nodes (MLN) after DSS treatment to assess potential inflammatory signatures among gut-draining immune cells (Figure 11A-B). Unlike in the colon, where *A. muciniphila* ameliorated *A. mucolyticum*-induced responses, we found that co-colonization instead appeared to blunt putative *A. muciniphila*-induced immune responses in the MLN. For example, MLNs from mice colonized with MC+*A. muciniphila* contained elevated levels of dendritic cells (DC) compared to those colonized by MC alone or MC+*A. mucolyticum*, and this effect was abrogated in mice co-colonized with MC+both *A. mucolyticum* and *A. muciniphila* (Figure 11C). These data imply that *A. mucolyticum* colonization may alter *A. muciniphila*-induced immune responses, and highlight the reciprocal, bi-directional nature of this microbial epistasis paradigm.

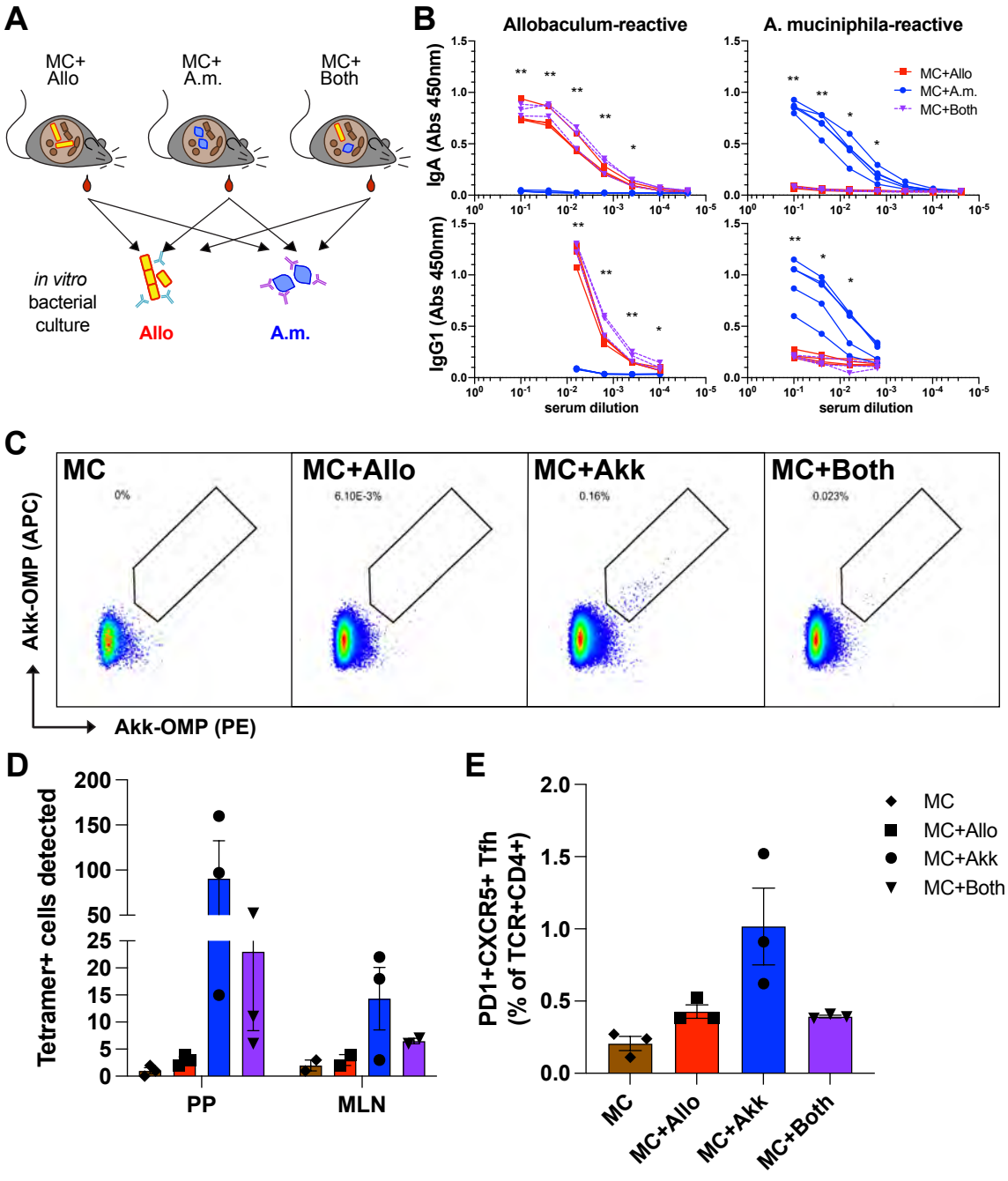

Figure 12. *A. mucolyticum* blunts *A. muciniphila*-induced B & T cell responses at steady state. (A) Schematic shows the experimental workflow for analyzing week 6 serum antibody binding to cultured bacterial cells. (B) Dilution curves show *A. muciniphila*-reactive and *A. mucolyticum*-reactive serum IgA & IgG1 from mice colonized with each microbiota (n=3-5). Welch's t-test was used to compare MC+A.m. to MC+Both at each dilution. * P<0.05, ** P<0.01. Representative data from one of N=4 independent experiments. (C) Representative FACS plots of MLN CD4$^+$ T cells stained with H2-K^b tetramers loaded with *A. muciniphila* outer membrane protein (OMP) peptide. (D) Quantification of tetramer-positive T cells from MLN and PP. (E) MLN follicular T helper (Tfh) cells.

2.2.D. Pairwise effects of *A. mucolyticum* and *A.muciniphila* on systemic antibody responses

Based on the observed effects of co-colonization on immune activation in the MLN after DSS treatment, we hypothesized that co-colonization may affect the development of *A. mucolyticum*-specific and *A. muciniphila*-specific immune responses in a reciprocal manner. Because both *A. mucolyticum* and *A. muciniphila* induce potent systemic IgG responses (Ansaldo et al., 2019), we next examined the effects of co-colonization on systemic antibody responses at steady state (Figure 12A). As expected, colonization with *A. muciniphila* or *A. mucolyticum* in the MC background elicited potent bacteria-specific serum IgG and IgA responses (Figure 12B). Despite the protective effects of *A. muciniphila* on *A. mucolyticum*-induced colitis, *A. mucolyticum*-specific antibody responses were unaltered after co-colonization. In contrast, co-colonization almost completely blocked the induction of *A. muciniphila*-specific serum IgA and IgG1 responses (Figure 12B). Overall, these data show that *A. mucolyticum* blunts *A. muciniphila*-induced systemic antibody responses and that co-colonization with *A. mucolyticum* and *A. muciniphila* alters the immune responses elicited by each organism in isolation.

2.2.E. Pairwise effects of *A. mucolyticum* and *A.muciniphila* on mucosal T cell responses

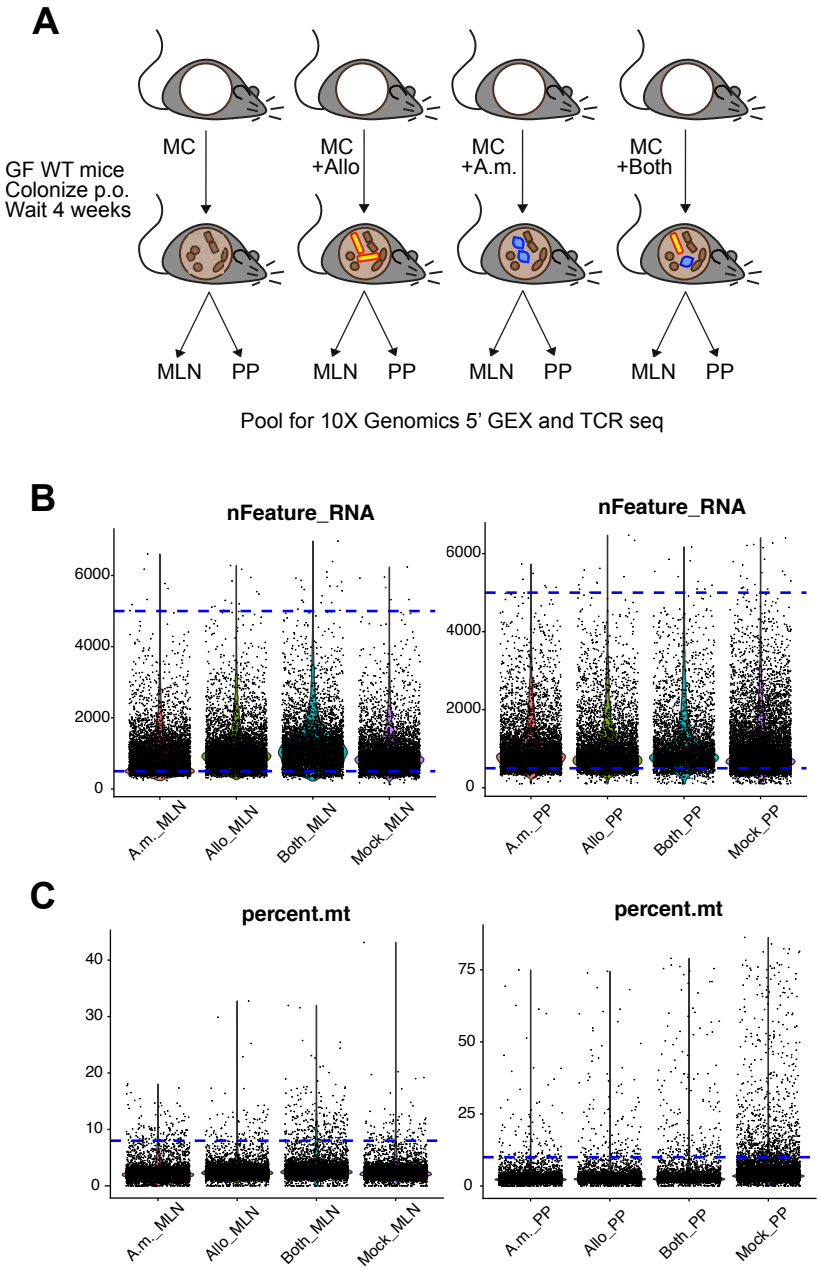

Figure 13. Strategy for assessing *A. mucolyticum*- and *A. muciniphila*-induced transcriptomic reprogramming in mucosal lymphoid tissues. (A) Experimental design for single cell RNA sequencing shown in Fig. 13-14. (B-C) Filtering of high quality cells that contained between 500-5,000 RNA features (B), and less than 8% of RNA features mapping to mitochondrial (mt) genome (C).

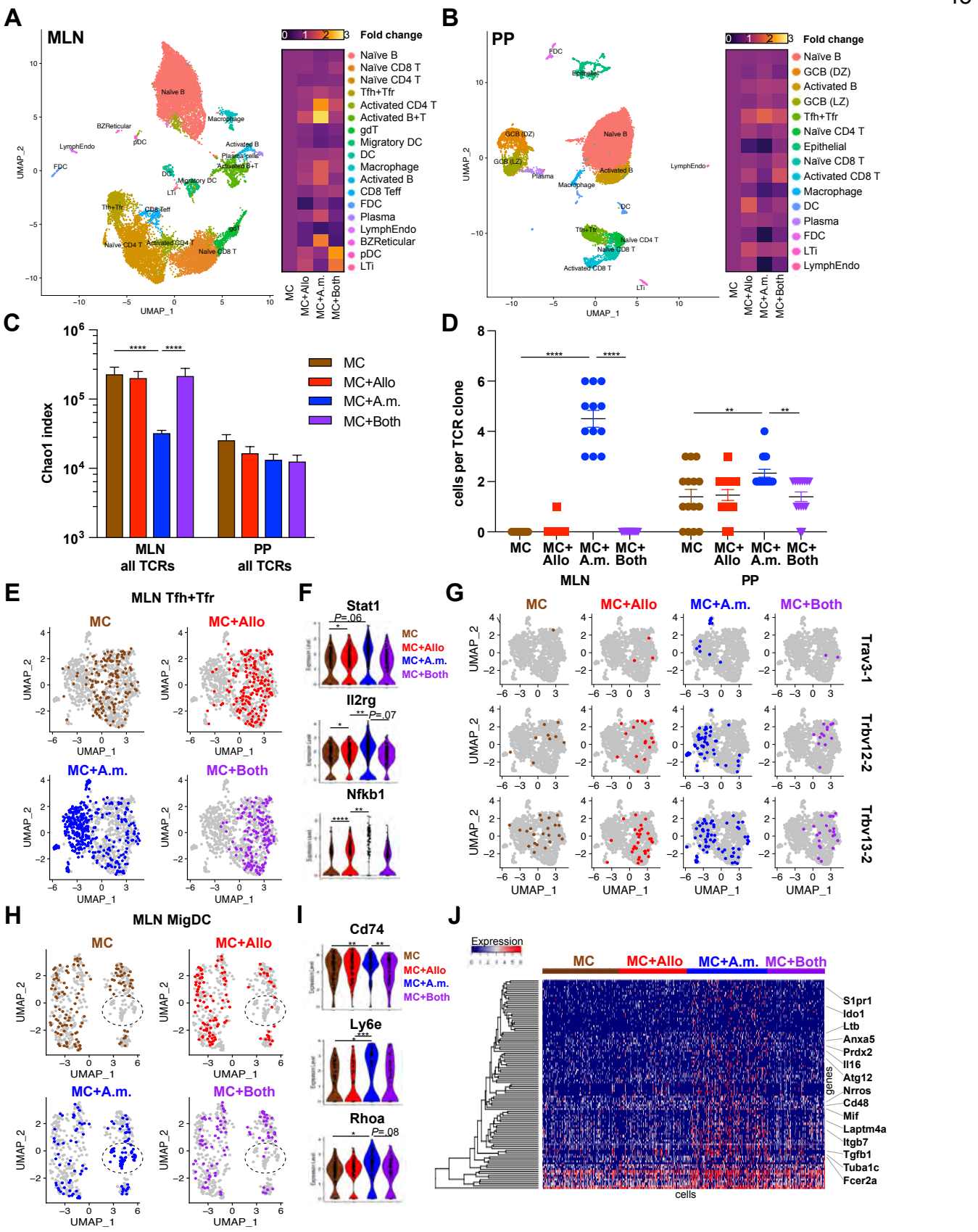

Figure 14. *A. mucolyticum* and *A. muciniphila* induce context-dependent transcriptomic reprogramming in mucosal lymphoid tissues. (A-B) Annotated UMAP dimensionality reduction plots of single-cell gene expression libraries, pooled by tissue (A, Mesenteric lymph nodes (MLN); B, Peyer's patches (PP)). Right, heatmap of each cell lineage frequency normalized to MC. (C) TCR repertoire diversity. (D) Top 12 most expanded clonotypes in MC+A.m. colonized mice and their corresponding silencing. (E,H) MLN Tfh+Tfr and MigDC were examined in isolation, re-clustered, and highlighted by microbiome. (F) Expression of key genes within Tfh+Tfr shown across microbiome. (G) Prominent TCR clonotypes within MLN Tfh+Tfr induced by MC+A.m. (H) MLN MigDC UMAP clustering. (I) Expression of key MigDC antigen presentation genes, including Cd74 (Ii, invariant chain). (J) Top 100 differentially expressed genes within MLN MigDC. Welch's t-test was used to compare gene expression across microbiome groups. * P<0.05, ** P<0.01, *** P<0.001.

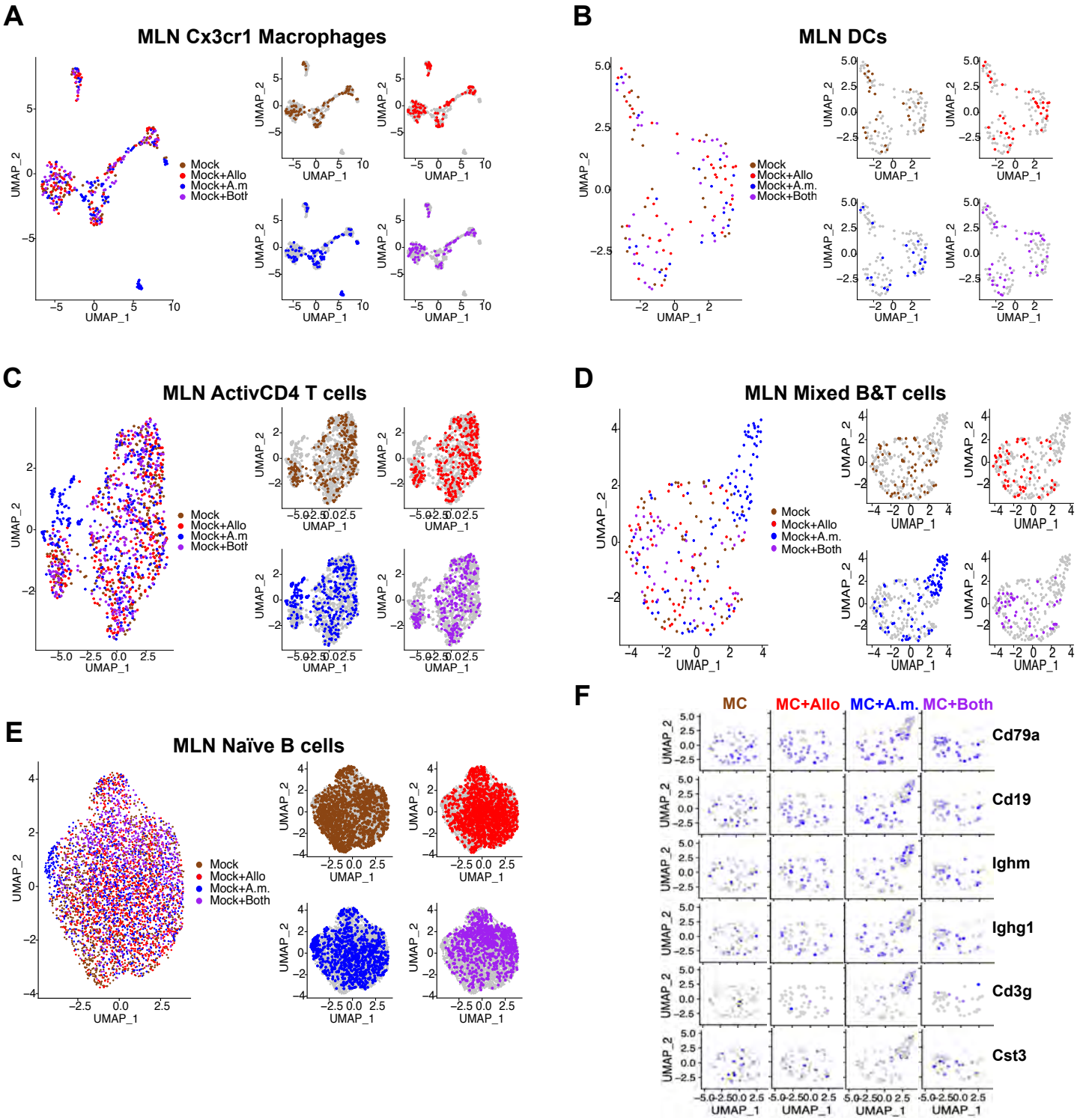

Figure 15. *A. mucolyticum* and *A. muciniphila* induce transcriptomic reprogramming in innate and adaptive MLN immune cells. Various MLN cell populations were subsetted by lineage and reclustered in isolation, shown as UMAP plots split by microbiome. (A) Cx3cr1^{+} macrophges, (B) DCs, (C) activated CD4^{+} T cells, (D) mixed B&T cells, (E) naïve B cells. (F) split feature plots showing the presence of both B & T cell markers in the cells shown in (D).

To further explore the individual and epistatic impacts of *A. mucolyticum* and *A. muciniphila* on host immune responses, we performed simultaneous single-cell RNA sequencing (scRNA-seq) and repertoire sequencing on mesenteric lymph node (MLN) and Peyer's patch (PP) cells from gnotobiotic mice colonized for four weeks with either MC alone, MC with *A. mucolyticum* or *A. muciniphila,* or MC with both *A. mucolyticum* and *A. muciniphila* (Figure 13A). We captured 4,391-10,306 cells per microbiota group, with 77.3-93.6% cells passing quality filters set to retain only viable cells with high-quality transcriptomes (Figure 13B-C). After data scaling, dimensionality reduction, and manual annotation of clusters based on conserved marker genes, we observed significant microbiota-dependent alterations in the relative abundance of diverse immune cell populations (Figure 14A-B). Furthermore, diverse innate and adaptive cell subsets also exhibited microbiota-dependent alterations in their global transcriptomes, as measured by differential gene expression analysis.

As expected, *A. mucolyticum* colonization induced significant alterations in the immunological milieu in both the PP and MLN as compared to mice colonized with MC alone, including notable increases in activated B and T cells, plasmacytoid dendritic cells, and lymphoid tissue inducer (LTi) cells in the MLN, and increased T follicular helper (Tfh) cells, dendritic cells, and LTi in the PP (Figure 14A-B). However, *A. muciniphila* colonization induced an even more dramatic immunological restructuring, particularly in the MLN (Figure 15). This reprogramming was characterized by increases in activated $CD4^+$ T cells and B

cells, as well as increases in plasma cells, macrophages, and B cell zone reticular cells. Remarkably, the majority of *A. muciniphila*-induced changes in the MLN were severely blunted upon co-colonization with *A. mucolyticum*, while *A. mucolyticum*-induced alterations were either unaltered or enhanced upon co-colonization (Figure 14A). *A. muciniphila*- and *A. mucolyticum*-induced alterations in PP cellularity were less dramatic overall than those in MLN and were characterized mainly by an increase in Tfh cells, which was unaltered by co-colonization. However, *A. mucolyticum* colonization alone also induced modest increases in DCs and LTi cells, which was at least partially blunted by co-colonization (Figure 14B). Together, these data suggest that co-colonization with *A. mucolyticum* and *A. muciniphila* reprograms the immune responses elicited by each organism on its own.

Finally, we leveraged our scRNA-seq and antigen receptor repertoire data to dissect the cellular mechanisms by which co-colonization with *A. mucolyticum* blunts the *A. muciniphila*-induced systemic antibody response. As expected, we observed that *A. muciniphila*-induced alterations in BCR repertoires in the MLN and PP were blunted by co-colonization. For example, activated B cells expressing κ5-43 and κ3-2 and plasma cells expressing κ8-27 were expanded in MLN from *A. muciniphila* colonized mice, but were nearly undetectable in co-colonized mice, as were *Ighg2b*-expressing PP germinal center B cells (Figure S3, S4). Since the systemic antibody response to *A. muciniphila* is reported to be T cell-dependent (Ansaldo et al., 2019), we next examined the activation and

clonal expansion of T cells in individually colonized and co-colonized mice, with a specific focus on Tfh cells. *A. muciniphila* colonization alone was associated with the expansion of global TCR repertoire clonality, emergence of specific clonotypes in both the MLN and PP, and the appearance of a unique population of Tfh cells in the MLN (Figure 14C-G). However, these responses were nearly completely blocked by co-colonization with *A. mucolyticum* (Figure 14C-G). Using MHC class II tetramers loaded with *A. muciniphila* peptides, we also observed a coordinate reduction in antigen-specific T cells in the MLN and PP in co-colonized mice compared to those colonized with *A. muciniphila* alone (Figure 12C-E).

These data suggest that *A. mucolyticum* may prevent the initial priming of *A. muciniphila*-specific T cells in the MLN, for example by blocking *A. muciniphila*-induced activation or migration of professional antigen presenting cells such as dendritic cells (DCs). Indeed, we found that *A. muciniphila* colonization elicited a unique population of migratory DCs (MigDC) in the MLN that exhibited enhanced expression of transcripts encoding antigen presentation machinery and activation markers, and the appearance of these cells was completely abrogated by co-colonization with *A. mucolyticum* (Figure 14H-J). Overall, these data suggest that *A. mucolyticum* may block *A. muciniphila*-specific systemic immune responses at the initial priming step, enforcing tolerance and confining any immunological activity against this gut commensal to the local mucosa.

2.3. Conclusions

Species belonging to genus *Allobaculum* are present in the gut microbiota of healthy humans, and are anti-correlated with *Akkermansia muciniphila*, leading us to conclude that this novel microbiota axis is directly relevant to human health and disease. While the latter taxon is far more prevalent in the general population, we highlight the detection of a newly characterized taxon in hundreds of subjects in these cohorts that may point to a risk factor for development of inflammatory bowel disease. Yet, in our animal studies, co-colonization with both *A. mucolyticum* and *A. muciniphila* provided protection from the inflammatory sequelae of *A. mucolyticum*-mediated acute colitis. We speculate that this interaction may be worth preclinical exploration as a means of IBD prevention.

Concurrently, in a reciprocal epistatic manner, co-colonization with both *A. mucolyticum* and *A. muciniphila* dampened *A. muciniphila*-induced innate and adaptive immune responses, indicating that the putative beneficial effects of *A. muciniphila* on metabolic outcomes or efficacy of cancer immunotherapy may be negated by the presence of other immunostimulatory taxa like *Allobaculum sps.* The open question remains whether in human clinical trials high doses of live biotherapeutics (LBT) containing *A.muciniphila* would overcome a relatively rare but perhaps potent colonization by *Allobaculum* strains. In either case, our data point to an aspect of the immunostimulatory human gut microbiota that has not been examined to date.

Reflecting more broadly, we conclude that this reciprocal epistatic modality might also be found in other pairs of immunostimulatory members of the human gut microbiota. We predict that these pairs of microbes will exert functional effects seen well beyond the mucosal tissues themselves, ranging from effects on the central nervous system to vaccine responses to core metabolic programs. This paradigm of microbial epistasis can be further explored in curated microbiome datasets to identify additional taxa that may illuminate our understanding of the host-microbe, or metaorganismal, interface, and potentially enable development of “precision probiotics.” These efforts to develop novel LBTs could be used to neutralize unwanted effects of inflammatory gut microbes or augment the desired beneficial functions of true symbionts. Advances in machine learning and deep neural network computing will likely empower the next generation of researchers to glean important insights from multi-omics data about the enormous multitude of possible pairwise interactions between strains that colonize the human gut. The authors humbly admit that while this *in silico* approach has been a tantalizing opportunity to consider, our technical and training limitations prevent us from stepping fully into these computational endeavors at the present moment.

2.4. Pitfalls, weaknesses, and alternative interpretations

As we began assessing various human microbiome hits that emerged from our *in vivo* discovery screen, one pitfall that emerged is the statistical “noise” present in

our meta-analysis of large-scale human microbiome data. Although the *A. muciniphila* lead that we pursued into mechanistic animal experiments seems to have led down a fruitful line of inquiry, we suspect that there is much more information to be extracted about additional microbial taxa that may interact epistatically with *Allobaculum sps.* in humans. Many of these taxa seem to be correlated in the human datasets to the same degree of statistical significance as does *A. muciniphila*, when examined by generalized linear modeling (GLM) approaches. However, they did not emerge from our *in vivo* gnotobiotic screen, which, importantly, tested interactions with our laboratory isolate of *Allobaculum mucolyticum*, whereas those strains spread throughout the general population likely exhibit genetic and phenotypic variability. Additionally, large-scale datasets come with their own complications, and a wide range of factors must be considered during data curation such as patient stratification, sample collection and processing, sequencing depth, and OTU picking.

Another weakness is our lack of mechanistic understanding of *A. muciniphila*-mediated protection against *A.mucolyticum*-induced colitis. Studies from European groups have identified Amuc_1100 as a bioactive protein from *A. muciniphila* that can mediate the same effects combating metabolic syndrome as does the live organism (Plovier et al., 2017). We haven't assessed whether purified Amuc_1100 is able to mitigate *A.mucolyticum*-mediated inflammation in mice, nor whether any other crude cell fraction is more active than another fraction (e.g., secreted molecule in the supernatant). Ongoing experiments seek

to assess whether administration of heat-killed *A. muciniphila* to *A. mucolyticum*-colonized mice exert the same immunoregulatory forces as does colonization with the live strain.

In trying to understanding the cellular and molecular mechanisms of microbial epistasis, one alternative interpretation of our data would be that the immunoregulatory effects of *A. muciniphila* in mitigating inflammation is achieved simply by reduction of *A. mucolyticum* CFU burden to a particular anatomical site (e.g., beyond the mucosal firewall). In this way the mechanism of protection could be explained much in the same way that antibiotic drugs can limit disseminated infection–by keeping the number of live CFUs in sensitive tissues as low as possible. Similarly, the effects of *A. mucolyticum* that limit induction of immune responses against *A. muciniphila* might also be based on exclusion of these cells or antigens from inductive sites. We have little data to support this speculation, however, and further experiments are needed to formally address these hypotheses.

Besides the above CFU-based interpretation, it's indeed possible that *A. muciniphila* might be producing high levels of short chain fatty acids (SCFAs), or inducing other members of the mock community to do so. This metabolite-based mechanism could reinforce epithelial barrier integrity, stimulate greater mucus production, and augment regulatory T cells (Tregs), all of which could contribute to the observed protective phenotype.

2.5. Potential solutions and future directions

With the wealth of sequencing data that's publicly available, we propose to continue meta-analysis of further human cohorts and to develop more rigorous statistical models to assess concordance of bacterial hits emerging from our xenografted human microbiota screen with their representation in real-world data. Furthermore, we are excited by the possibility of identifying and exploring additional novel pairwise epistatic interactions in the human microbiota using this same general approach. This microbial epistasis may prove to be operational in a range of other phenotypic scenarios outside the gut mucosa.

In order to gain some insight into mechanisms of host susceptibility to *A. mucolyticum*-driven inflammation and rescue by *A. muciniphila*, we plan to examine intestinal epithelial cell dynamics using transcriptomic approaches and functional assays *in vitro*. We suspect that, although we have no evidence pointing to toxicity or cell death modalities under the influence of *A. mucolyticum*, there may be other important programs of activation or antigen presentation that may affect ensuing responses by innate immune cells and subsequent recruitment and activation of lymphocytes. The groundwork laid by many other labs makes this aspect of the project somewhat less exciting to us than the unexpected finding about microbial epistasis. Disentangling the bacterial mechanisms of action upon murine hosts will be a true challenge, as detailed in

Chapter 3. Although we can start with some bacterial transcriptomic approaches to examine what networks of genetic regulation are occurring in these microbes under various colonization conditions, we expect that there will be a lot of transcripts changing coordinately in mice co-colonized with both *A. mucolyticum* and *A. muciniphila.* The challenge will be deciphering which of these pathways is most meaningful to the outcome of the host animal as opposed to those transcriptional changes that are modulated but irrelevant, for example those enabling functions of core carbohydrate metabolism. To complement this *ex vivo* approach, we also plan to examine bacterial broth cultures and co-cultures that will aid in separating the direct strain-vs-strain interactions in sterile broth from the additional host signals coming from the complex murine gut environment.

To assess the possibility that CFU penetration through, or exclusion from, the mucosal firewall is a mechanism governing phenotypic outcomes to these commensal strains, we plan to extensively characterize the absolute quantity of these microbes in various tissue compartments by careful dissections followed by culture dependent methods for CFU enumeration, absolute quantitative PCR with genome-specific primers, as well as metagenomic sequencing with absolute-quantification spike-in reagents. We admit that while this possibility is not the most exciting explanation of reciprocal epistasis, it is still centrally important to precisely determine the extent to which CFU quantity explains inflammatory severity. If this barrier regulation phenomenon were the case, our paradigm would need to shift away from a model of epistasis towards a more traditional

understanding of *A. mucolyticum* as a classic opportunistic pathogen that simply relies on getting “a foot in the door”. Nonetheless, we have found both fulfillment and mystery while characterizing this novel microorganism and plan to report our findings publicly either way for the collective knowledge of the field.

Chapter 3: Exploration of immunogenic molecular properties of *Allobaculum mucolyticum*

3.1. Introduction

Microbiome researchers face an enormous challenge that will likely remain for several more decades at the present rate of innovation. The genomes of most human gut commensals are largely a “black box” because the majority of their detectable ORFs are unannotated (Kumar et al., 2016; Li et al., 2018). With little homology to characterized proteins and RNAs, the molecular functions of gut commensal microbes leave researchers little choice but to turn to traditional means of characterizing genes and their protein products or develop new technologies altogether.

The genome of *A. mucolyticum* falls into this same category, with 52% of its ORFs being unannotated by RAST. Over the course of this study, we have split our time somewhat unevenly between characterizing the model system in gnotobiotic mice and searching for the functional molecular properties endowed in this unique pathobiont. Ultimately, we search for the molecular basis of this strain’s potent effects in order to (1) illuminate more of the uncharacterized microbiota “dark matter”, (2) understand the basis by which these strains may contribute to the etiology of inflammatory bowel disease, (3) grasp the specific antigenic effects by which *A. mucolyticum* and *A. muciniphila* exert epistatic effects on the mammalian immune system, and (4) deepen our knowledge of this

poorly characterized taxon that may have other undiscovered functional modalities in human subjects.

An additional aspect of this challenge lies in the fact that many members of the human gut microbiota are difficult to cultivate and manipulate genetically. Even if present-day genome annotation technologies were vastly improved, we would still likely struggle to produce mutant strains with typical reverse genetics approaches. Leaders in the field have only just begun to make progress in manipulation of Gram-positive commensals, with family *Clostridiaceae* showing the greatest promise to date (Guo et al., 2019). In order to characterize novel genes and their protein products that impact host phenotype, we must first gain further basic understanding of these taxa and the mechanisms that render them so intractable.

Nonetheless, a small handful of studies have characterized commensal-derived antigens and begun to forge a path forward into mechanistic understanding of commensal antigens and their effects on the host. Notable examples of these microbial molecules that induce murine immune responses include *Bacteroides fragilis* polysaccharide A (PSA), P3340 and P4990 from segmented filamentous bacteria (SFB), and Am3735 and Am3740 from *Akkermansia muciniphila* (Ansaldo et al., 2019; Ladinsky et al., 2019; Mazmanian et al., 2005; Yang et al., 2014). By various mechanisms, these molecules lead to robust activation and clonal expansion of T lymphocytes even though they don’t accompany classical

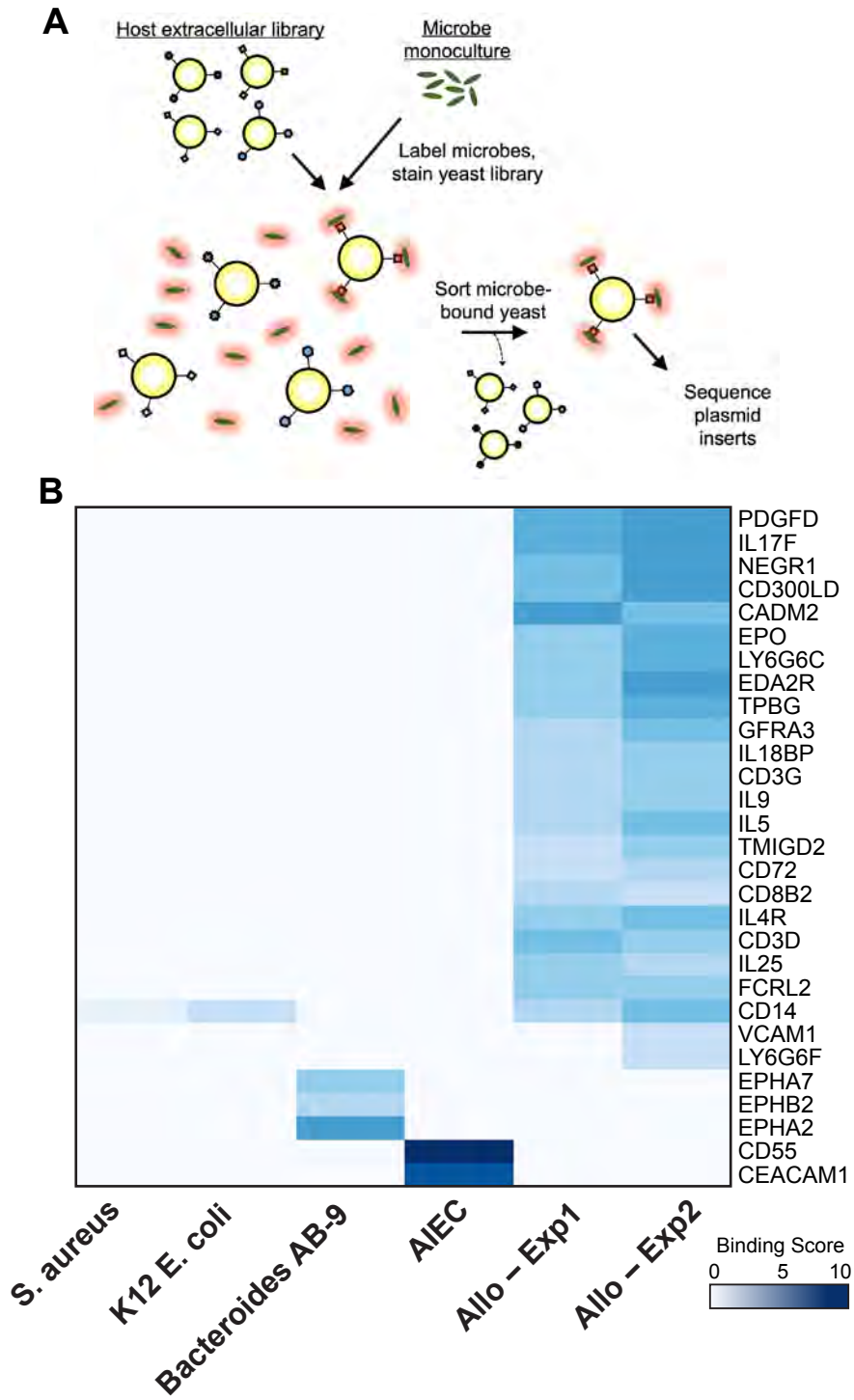

Figure 16. *A. mucolyticum* binds to numerous cell surface and secreted proteins. (A) Diagram of high-throughput yeast display screening platform and experimental workflow to assess bacterial binding. (B) Curated heatmap of top human proteins bound most strongly by bacterial isolates (columns), including two independent experiments with *Allobaculum mucolyticum*. AIEC: Adherent invasive *E. coli.*

infection. These findings have left researchers with a number of outstanding questions about the rules governing the host-microbiota interface.

In this work we have undertaken studies to identify the means by which *Allobaculum mucolyticum* exerts forces on human and mouse cells that lead to activation, inflammation, and ensuing immune responses. Despite the substantial challenges discussed above, these experiments are beginning to provide insight into the unique traits of this novel commensal taxon that are likely operational in *Allobaculum*-colonized human subjects around the world.

3.2. Results

3.2.A. *A. mucolyticum* binds to numerous cell surface and secreted proteins

Utilizing a high-throughput screening technology developed by a recent alumnus of the lab, Dr. Connor Rosen, we sought to ascertain the scope of protein-protein interactions between cultured *A. mucolyticum* and ~2,000 human surface and secreted proteins, known as the “exoproteome” (Gupta et al., 2020). Briefly, yeast display of exoproteome proteins from barcoded expression vectors and co-incubation with bacteria of interest allows for enrichment of binding partners by magnetic cell separation (MACS), followed by plasmid DNA isolation, PCR, next-generation sequencing, and quantification of the barcoded plasmids compared to original unbound yeast library (Figure 16A). In two independent experiments with

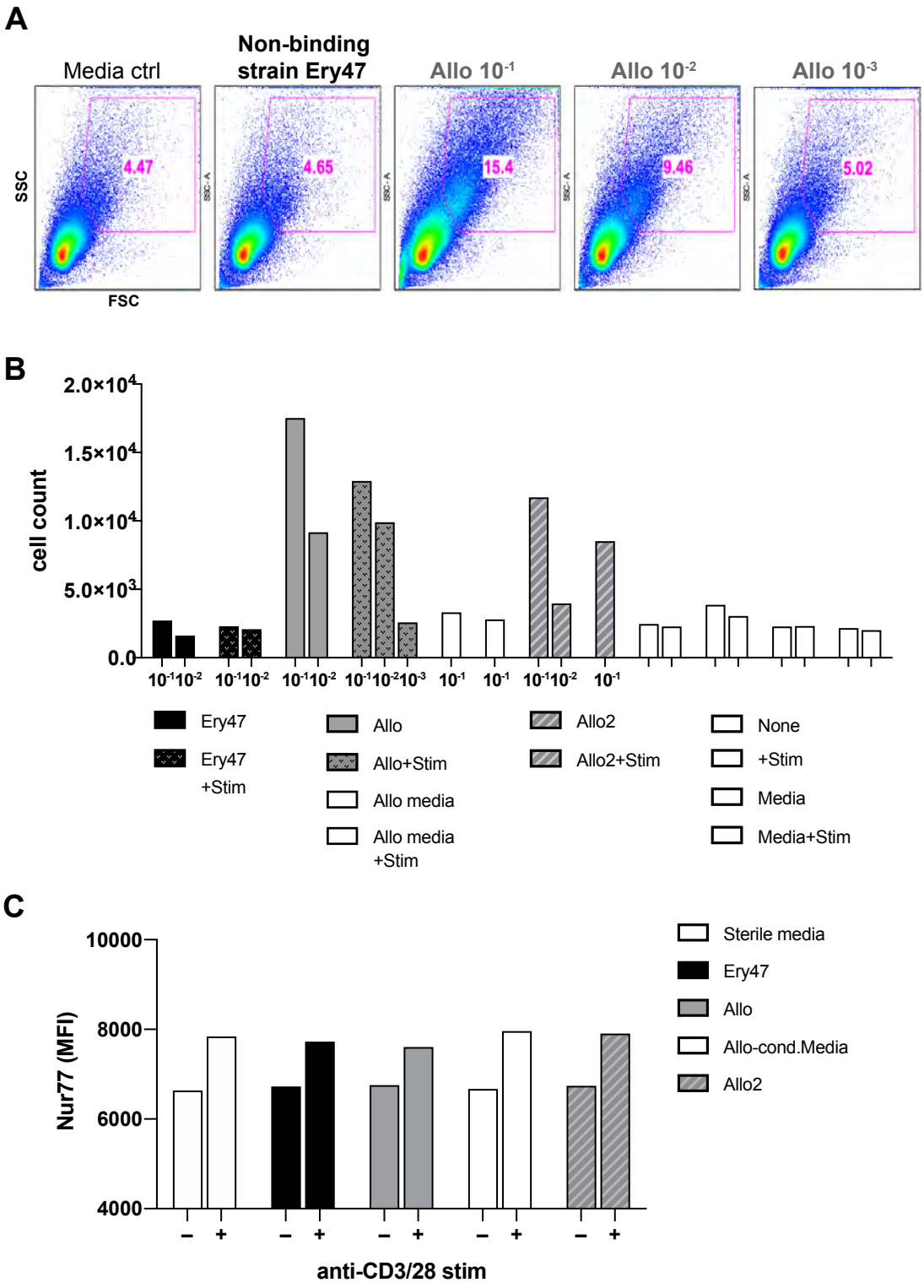

Figure 17. *A. mucolyticum* binds Jurkat T cells but fails to activate TCR. (A-B) Representative FACS plots and quantification of T cell-bacteria binding assay. Cells were stimulated with anti-CD3/28 for 2h before staining and analysis. (C) Nur77 expression.

A. mucolyticum cultured *in vitro*, we found that this novel pathobiont bound to numerous human proteins, including CD3G and CD3D, more so than any control bacteria (Figure 16B). We found this binding pattern intriguing not only because of its breadth, but also due to the presence of several proteins implicated in immune responses, including cytokines, cytokine receptors, coreceptors, and adhesins. Given our *in vivo* evidence for *A. mucolyticum's* potential for activation of both innate and adaptive immune cells, we wondered how much of the cellular phenotypes might be explained by this extensive exoproteome binding profile. Thus, these screening results prompted us to follow up on certain hits in cell-based validation studies.

3.2.B. *A. mucolyticum* binds to T cells but does not exhibit superantigen activity *in vitro* nor induce clonal expansion *in vivo*

Using the Jurkat human T cell line as an experimental system, we asked whether the predisposition of *A. mucolyticum* to bind human exoproteins on yeast would enable it to bind to and activate live T cells. Using an increase in light scatter as a proxy for bacterial binding, as well as later confirmation using fluorescently labeled bacteria, we found that *A. mucolyticum* binds T cells to a substantial degree more than closely related Gram-positive commensal strain Ery47 (Figure 17A). This not only confirmed the utility of our high-throughput protein interaction screening platform, but opened up a new *in vitro* model system for probing host-microbe interactions at the cellular level. Notably, these data also revealed that

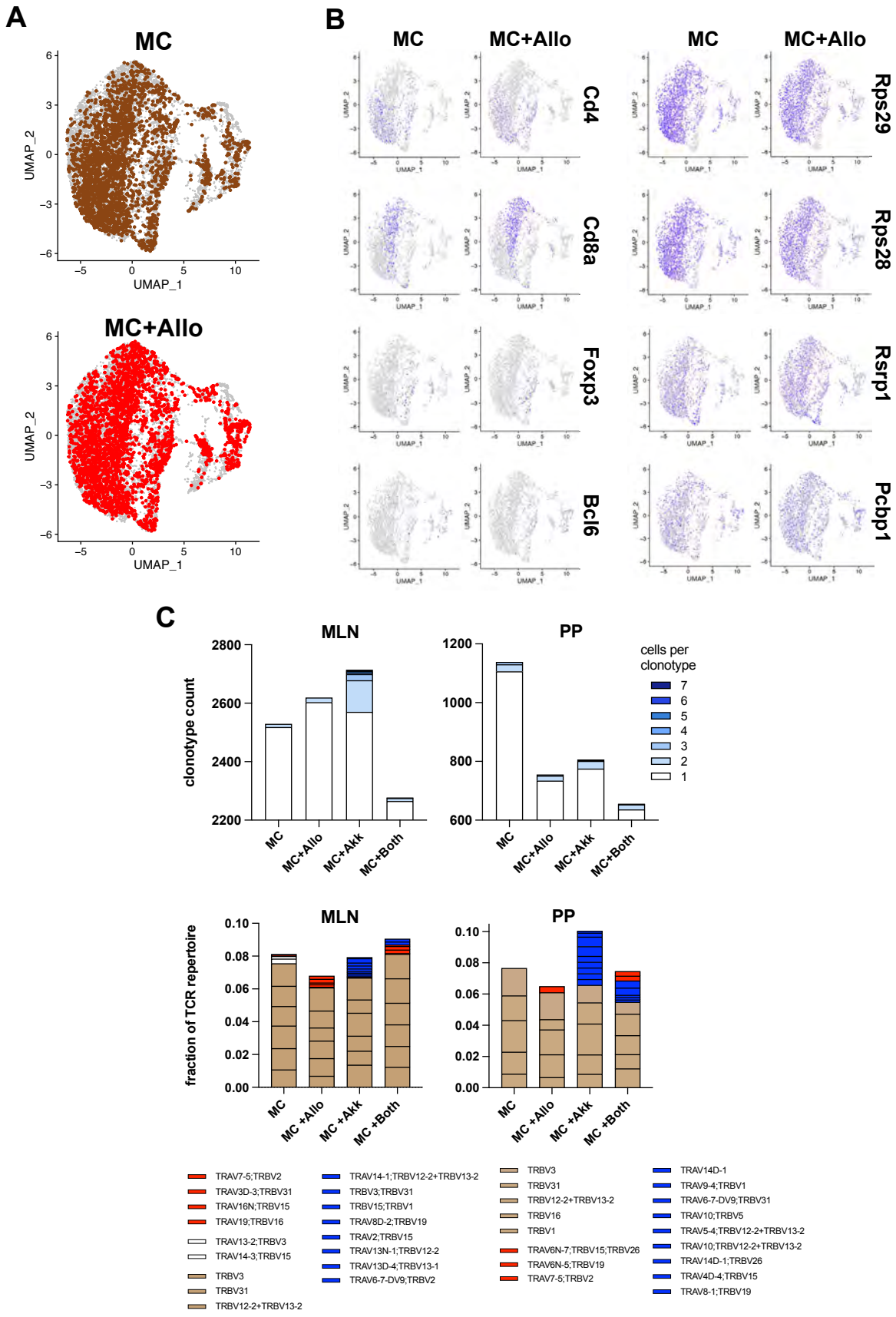

Figure 18. *A. mucolyticum* fails to induce dramatic transcriptional alterations or clonal expansion in MLN or PP T cells. (A) UMAP dimensionality reduction plots of MLN T cells analyzed by scRNAseq. (B) Feature plots of MLN T cells for major lineage markers (left) and top 4 most differentially expressed genes from MC T cells to MC+Allo T cells (right). (C) TCR repertoire analysis showing clonal expansion.

the binding capacity of *A. mucolyticum* is not due to a secreted molecule in the culture supernatant, but rather the cell surface itself (Figure 17B).

Pathogenic bacteria like *Staphylococcus aureus* are known to produce enterotoxic molecules called superantigens (SAgs) that cross-link TCR interactions and result in extensive T cell activation and cytokine storm. We next examined whether *A. mucolyticum* leads to direct T cell activation, like a classical superantigen. Our *in vitro* cellular assays revealed that although there is evidence of *A. mucolyticum* binding to T cells, they fail to activate TCR, as measured by Nur77 staining (Figure 17C). We also performed the same assay using fresh human PBMCs and similarly saw binding by *A. mucolyticum* but no T cell activation. The increase in forward scatter (FSC) in T cells incubated with *A. mucolyticum* tempts us to speculate that there is some degree of activation-induced cell death or apoptosis occurring, but we did not formally test this using vital dyes like propidium iodide, or annexin V staining of membrane phosphatidylserine.

To examine the possibility that *A. mucolyticum* might influence T cell activation and TCR clonality *in vivo*, we also undertook a reanalysis of our single-cell RNA and TCR repertoire sequencing data. Unsupervised clustering and differential expression analysis of all MLN T cell lineages in MC+Allo- versus MC-colonized mice revealed that there was only subtle transcriptional alteration of T cells by *A. mucolyticum* at baseline (Figure 18A-B). Even the top-ranked differentially

expressed genes across these microbiota conditions yielded few notable differences in the MLN T cell compartment (Figure 18B). Turning to the TCR repertoire, quantification of T cell clones that were most represented in the MLN and PP of MC+Allo-colonized mice revealed no significant expansion of T cells in either tissue after 4 weeks of colonization compared to MC-colonized mice (Figure 18C). We highlight the stark contrast between this meager effect and that of colonization with MC+*A. muciniphila,* which led to marked clonal expansion of MLN T cells at baseline (Figure 12E, 12G, 18C). Together, these *in vitro* and *in vivo* data reveal little support for the idea of *A. mucolyticum* as a direct activator of murine or human T cells, despite some measure of binding. We suspect that long chains of cultured *A. mucolyticum* cells, as seen in Figure 2B, may exhibit high avidity interactions more than specific receptor-ligand binding as suggested by our exoproteome screen.

3.2.C. Exploration of immunodominant *A. mucolyticum* antigens remains inconclusive

Recalling our earlier results wherein *A. mucolyticum* induces serum antibody responses in mice at baseline, we sought to use these antibodies as a potential tool for investigating immunodominant *A. mucolyticum* antigens. We conducted classic immunoprecipitation experiments, with the hopes of exploring antigens in crude lysate bound by serum Ig from seroconverted MC+Allo mice. Despite immunoblots that show clear binding of serum IgA and IgG to a high molecular

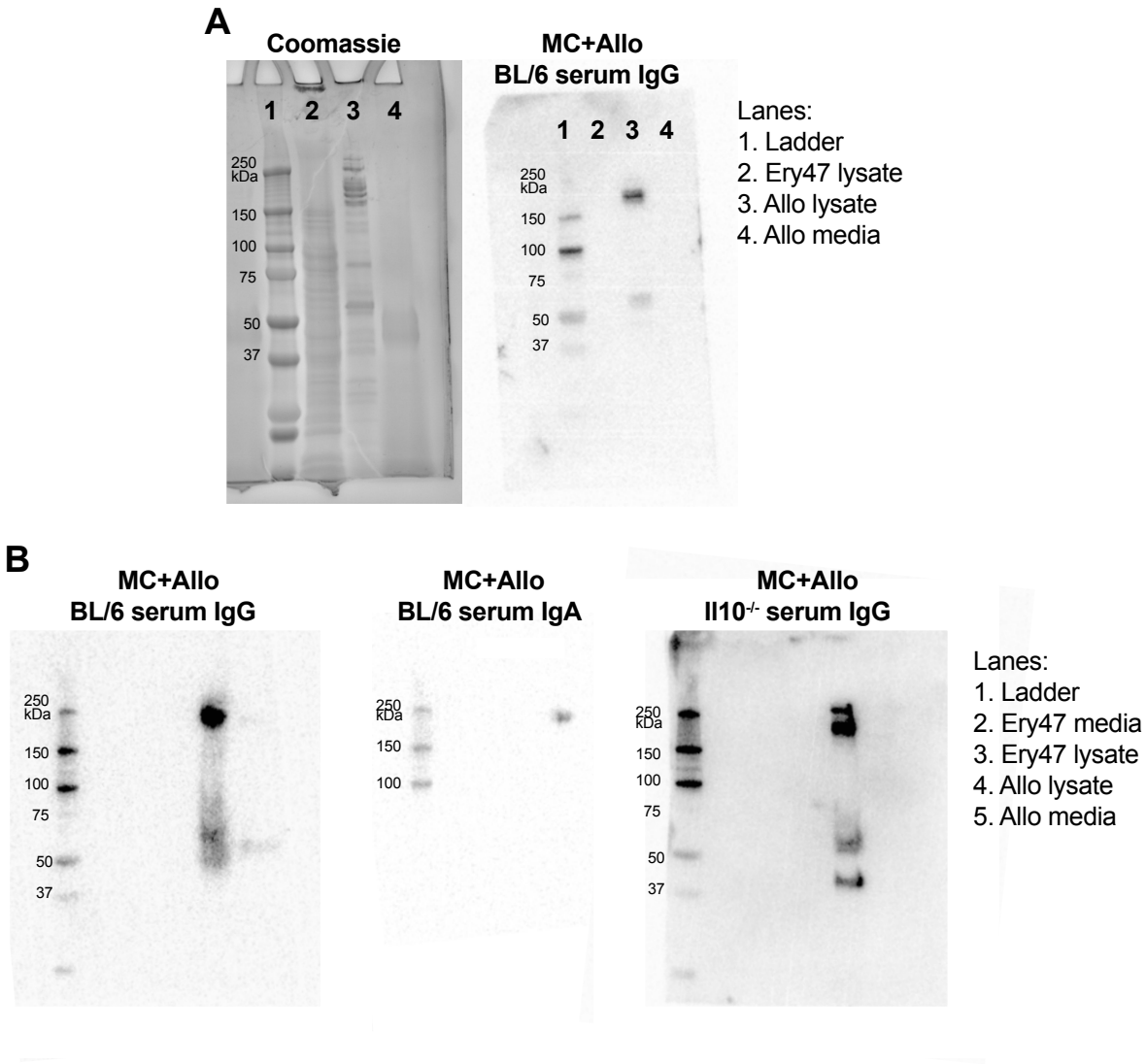

Figure 19. Western blots for immunodominant *A. mucolyticum* protein antigens reveal two major IgG-bound species: ~200kDa and ~60kDa. (A) SDS-PAGE analysis of bacterial lysates, stained for total protein content with Coomassie blue (left) and blotted and probed with polyclonal week 7 gnotobiotic mouse antiserum, before detection of mouse IgG (right). (B) Western blots of bacterial lysates using week 8 serum from WT BL/6 gnotobiotic mice or week 8 Il10$^{-/-}$ mice.

weight (>200 kDa) protein, our efforts to use untargeted LC/MS to identify these antigens were uninterpretable due to high levels of murine proteins (Figure 19A-B). More curated proteomics approaches may help uncover the nature of these immunodominant antigens in subsequent experiments.

Separately, we used an *in silico* approach in collaboration with the Ramnik Xavier lab called BOTA: Bacterial Origin T cell Antigen predictor to identify I-A^b-restricted peptides that may be immunogenic using a deep neural network-based algorithm (Graham et al., 2018). Using the *A. mucolyticum* genome sequence as input, this software package returned 108 peptide hits, of which we curated the top 40 (Figure 20). Ideally, we plan to synthesize these peptides and use a pooled approach to begin screening T cells *ex vivo* from gnotobiotic mice colonized with *A. mucolyticum*. Rather than direct TCR activation by bacterial binding alone, we suspect that peptide-MHC presentation by professional antigen presenting cells will yield more suitable conditions for evaluating these candidate antigens for their ability to restimulate T cells *ex vivo*. In this way we can begin narrowing in on immunodominant antigens that might serve as potential targets in preclinical efforts to neutralize the inflammatory properties of *A. mucolyticum* in at-risk patients.

3.2.D. Loss-of-function *A. mucolyticum* non-binding mutants exhibit a range of altered traits

#peptide	gene_name	gene_annotation	score	gene_start	gene_stop	protein_size	strand	pep_start	pep_stop	pep_len
LTYAELAAAKAEEA	>contig_114_2	DUF4981 domain-containing protein [Lachnoclostridium sp. An181]	0.987	854	6154	1767	1	5062	5106	44
FDAAMTNAA	>contig_169_12	DUF4981 domain-containing protein [Tyzzerella nexilis]	0.966	11423	19090	2556	1	7315	7344	29
VQSVSVYLA	>contig_238_1	hypothetical protein [Ileibacterium valens]	0.958	3	1346	448	1	367	396	29
WSAVYPNAGAV	>contig_78_30	beta-galactosidase [Lachnoclostridium sp. An181]	0.935	38768	43783	1672	-1	3514	3549	35
FVTPASVLAA	>contig_52_5	DUF4981 domain-containing protein [Ruminococcus flavefaciens]	0.916	4402	5424	341	1	37	69	32
WSEWTTAPA	>contig_17_9	leucine-rich repeat domain-containing protein [Denitrobacterium detoxificans]	0.912	15784	19083	1100	-1	2413	2442	29
LYNPSASKS	>contig_29_2	alpha-glucosidase [[Clostridium] spiroforme]	0.910	921	3989	1023	1	2920	2949	29
LIDPNVLDS	**>contig_154_11**	**hypothetical protein [Lachnospiraceae bacterium NE2001]**	**0.894**	**20384**	**27124**	**2247**	**1**	**1186**	**1215**	**29**
FGSAKSSAA	>contig_133_12	hypothetical protein [Lachnoclostridium sp. An181]	0.885	14110	18708	1533	1	4462	4491	29
LQLPAPAFA	>contig_197_1	hypothetical protein B5G40_16410 [Flavonifractor sp. An9]	0.882	669	5255	1529	1	2527	2556	29
ASSPAANAQ	>contig_131_21	hypothetical protein [Allobaculum stercoricanis]	0.873	32752	35724	991	-1	2272	2301	29
FSSPQTAAA	>contig_187_11	TPA: hypothetical protein [Erysipelotrichaceae bacterium]	0.866	16220	18040	607	1	877	906	29
EEAPADSSASSSEA	>contig_99_14	hypothetical protein [Allobaculum stercoricanis]	0.859	13948	15333	462	-1	367	411	44
WKLNVAEAA	>contig_93_1	Alpha-L-fucosidase [uncultured Ruminococcus sp.]	0.854	670	3705	1012	1	2446	2475	29
FSLSSPVES	>contig_139_15	LPXTG-motif cell wall anchor domain-containing protein [[Clostridium] cocleatum]	0.852	13496	18793	1766	1	2110	2139	29
EYGPAVISY	>contig_20_17	Papain family cysteine protease [uncultured Ruminococcus sp.]	0.839	19535	22567	1011	-1	718	747	29
FAASPVRYA	>contig_87_20	patatin-like phospholipase family protein [Ileibacterium valens]	0.816	26824	28431	536	1	403	432	29
EEGSAAADS	**>contig_142_24**	**putative uncharacterized protein [Eggerthella sp. CAG:1427]**	**0.814**	**29698**	**35526**	**1943**	**-1**	**4723**	**4752**	**29**
AKAPQAEEA	>contig_117_4	DUF4091 domain-containing protein [[Clostridium] cocleatum]	0.799	3610	6879	1090	1	64	93	29
FSASSKTAA	>contig_167_1	hypothetical protein [Ileibacterium valens]	0.798	21	1292	424	-1	787	816	29
PEEPAALPV	>contig_137_4	hypothetical protein [Robinsoniella peoriensis]	0.783	7274	12574	1767	-1	1252	1281	29
LSAADYLEAAQTAIA	>contig_16_17	xylosidase/arabinofuranosidase [Blautia marasmi]	0.782	18517	24336	1940	1	5641	5688	47
LSAAIYSRA	>contig_10_17	carbohydrate-binding domain-containing protein [Larkinella rosea]	0.782	17672	19402	577	1	508	537	29
IAYSSVSAS	>contig_57_9	Uncharacterised Sugar-binding Domain [[Clostridium] cocleatum]	0.772	10013	10726	238	-1	307	336	29
FGGVVPEVA	>contig_150_8	tRNA (adenosine(37)-N6)-threonylcarbamoyltransferase complex transferase subunit TsaD [Allobaculum stercoricanis]	0.768	6002	7021	340	1	115	144	29
EYGPASISYAASSQS	>contig_137_16	papain family cysteine protease, partial [[Clostridium] methylpentosum DSM 5476]	0.755	24181	26130	650	-1	157	204	47
WADPIANIP	>contig_228_11	hypothetical protein [Allobaculum stercoricanis]	0.738	13005	14621	539	1	865	894	29
YKSAVASQSSFA	>contig_70_1	LPXTG cell wall anchor domain-containing protein [[Clostridium] spiroforme]	0.726	373	4641	1423	-1	4072	4110	38
FVLYSYTRS	>contig_109_17	hypothetical protein B5F73_03870 [Olsenella sp. An270]	0.713	18239	19378	380	-1	823	852	29
FQGITAYSASQL	>contig_17_11	BspA family leucine-rich repeat surface protein [Listeria monocytogenes]	0.700	20292	22157	622	-1	154	192	38
FRSYYAGTT	>contig_150_10	glycoside hydrolase family 2 [Lachnospiraceae bacterium V9D3004]	0.689	9064	13944	1627	1	2068	2097	29
YIYTSAASAA	>contig_205_7	DUF4968 domain-containing protein [Collinsella aerofaciens]	0.673	10111	16023	1971	-1	1828	1860	32
ITSYSAALD	>contig_249_28	BspA family leucine-rich repeat surface protein [Bifidobacterium pseudolongum]	0.666	30133	32565	811	1	1429	1458	29
GISASYSPY	**>contig_131_2**	**TIR domain-containing protein [Lachnospiraceae bacterium XBD2001]**	**0.652**	**609**	**4049**	**1147**	**-1**	**895**	**924**	**29**
VVLPASGPT	**>contig_231_11**	**filamentous hemagglutinin N-terminal domain-containing protein [Arcobacter sp. LPB0137]**	**0.652**	**14691**	**17381**	**897**	**-1**	**1846**	**1875**	**29**
VTTYAPAQV	>contig_137_3	hypothetical protein [Robinsoniella peoriensis]	0.649	2756	6937	1394	-1	3010	3039	29
RVGSSASPS	>contig_225_1	hypothetical protein [Ileibacterium valens]	0.646	2	1675	558	-1	1459	1488	29
WNSKAALES	**>contig_163_27**	**serine/threonine-protein kinase [Clostridiales bacterium CHKCI006]**	**0.639**	**36389**	**38398**	**670**	**-1**	**1720**	**1749**	**29**
FEVLTTYPS	>contig_154_6	serine/threonine protein kinase [Ruminiclostridium papyrosolvens C7]	0.622	12782	14725	648	-1	1177	1206	29
EKGASYTLY	>contig_16_24	hypothetical protein BN3662_02799 [Clostridiales bacterium CHKCI006]	0.620	37970	38689	240	1	547	576	29

Figure 20. Immunogenicity prediction by BOTA (DOI: 10.1038/s41591-018-0203-7) reveals *A. mucolyticum*-derived peptide hits that may be MHC class II-restricted antigens for activation of murine $CD4^+$ T cells. Peptides highlighted in blue bold originate from proteins predicted to be transmembrane proteins by TOPCONS (https://topcons.cbr.su.se/).

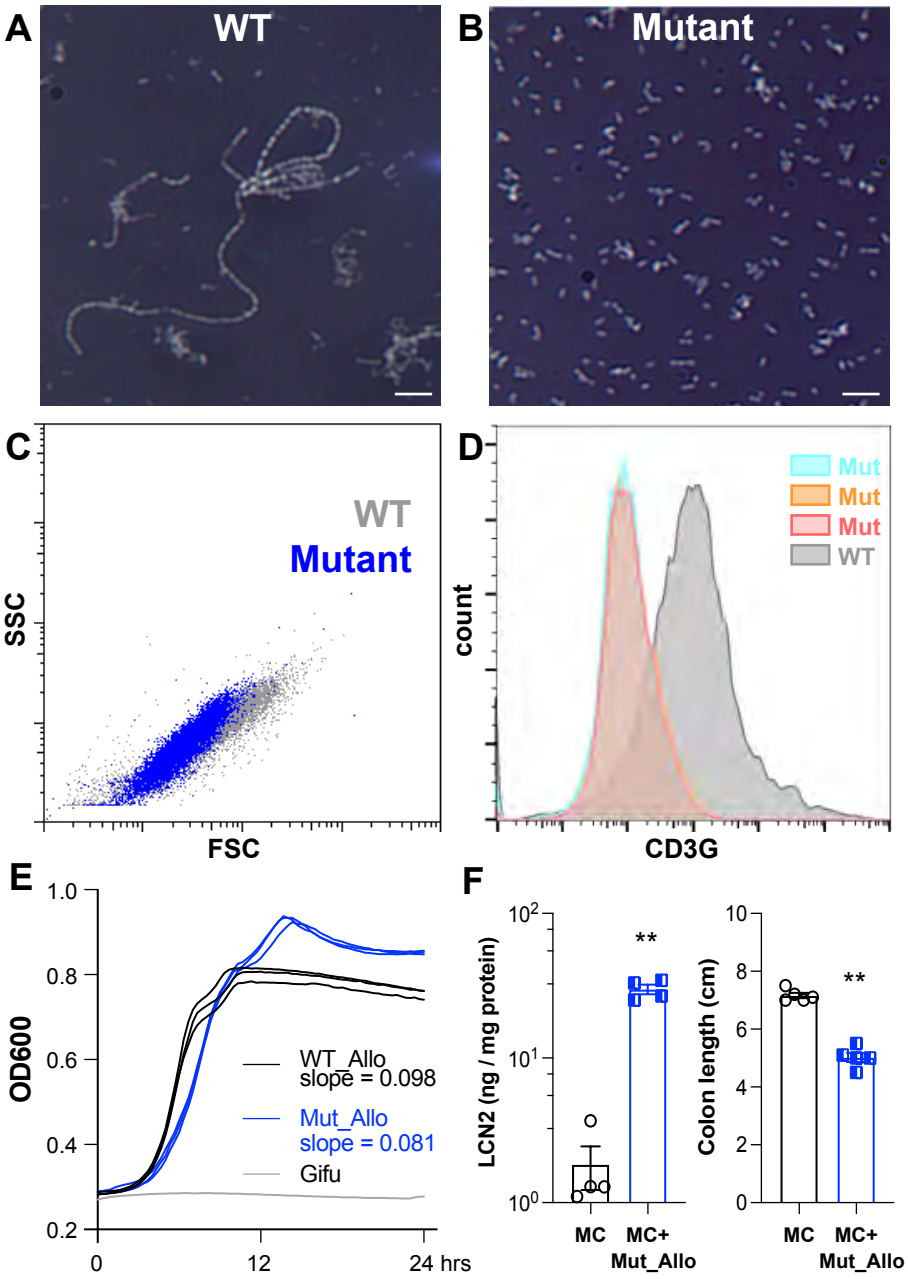

Figure 21. Mutant *A. mucolyticum* strain selected for loss of host binding still exacerbates colitis. (A-B) phase contrast microscopy of negative stained (A) parental WT and (B) Mutant *A. mucolyticum* in vitro. Scale bars, 5µm. (C) FACS scatter plot of WT and mutant *A. mucolyticum*. (D) FACS histogram of WT and mutant *A. mucolyticum* binding to recombinant human CD3G. (E) Growth curves of WT and Mutant *A. mucolyticum in vitro* and estimation of logarithmic phase growth rates. (F) Fecal lipocalin (LCN2) on DSS d3, and colon length at euthanasia on d6.

Lastly, to complement our approaches that have specifically followed the trail of B cell and T cell antigens from a host-centric perspective, we also performed experiments using a random mutagenesis forward genetics approach. After treating *A. mucolyticum* cells with an LD50 dose of ethyl methanesulfonate (EMS) to induce a substantial mutation load and recovering viable cells, we subjected this mutant library to negative selection for host receptor binding. Using a panel of 3 different recombinant human exoproteins that emerged from our high-throughput screening, we sorted a pool of triple-negative loss-of-function clones by FACS and spread the bacteria on agar for single colonies. We picked single colonies in order to characterize these *A. mucolyticum* mutants, and observed a morphological defect in 6 of the clones, wherein the enchained growth morphology of WT *A. mucolyticum* was lost (Figure 21A-C). We again confirmed that these cells exhibit loss of CD3G binding by FACS, and ensured they were as viable *in vitro* as their WT counterparts (Figure 21D-E). These results led us to speculate that this feature of *A. mucolyticum* cellular aggregation may perhaps lead to its inflammatory properties *in vivo.*

To test this hypothesis, we colonized WT gnotobiotic mice with MC+Mutant *A. mucolyticum* (Mut_Allo) or MC alone and induced acute colitis by administering DSS *ad libitum* in drinking water. As was the case with the parental strain of *A. mucolyticum*, we observed that mice colonized with MC+Mutant *A. mucolyticum* were profoundly more susceptible to DSS-induced inflammation than control MC-colonized counterparts (Figure 21F). From these results we conclude that neither

bacterial aggregation nor CD3G binding is the essential microbial feature leading to host inflammation, but further experiments to characterize the genetic lesions carried by these mutant strains may reveal other important aspects of the host-microbe interface.

3.3. Conclusions

From this slew of results it would be unwise to make many bold conclusions or claims about the true molecular basis of *A. mucolyticum's* immunostimulatory effects. Rather, as has been the case with microorganisms through the centuries, the mechanism by which this novel taxon incites a response from mammalian hosts remains elusive. We take this as an invitation to continue pressing forward with our scientific endeavors and pursuing those central questions that defy easy explanation: does this bacterial strain possess molecular machinery similar to that of a pathogen? How does this strain help us understand more deeply the classifications and mechanisms of interaction between commensals and their hosts? Despite the challenges faced in this arm of the study, we have learned a few things about the features and activities of this remarkable strain.

From our cellular assays and immunoprecipitation studies, we conclude that the active component of *A. mucolyticum* that activates adaptive immunity is unlikely to be a secreted protein, such as an exotoxin. We saw no activity or interaction in the cell-free supernatant from *A. mucolyticum* cultures, leaving us to follow the

trail of cell wall and cell membrane-associated molecules. This is in contrast to bona fide pathogens that secrete potent bioactive toxins. Neither in cell culture nor in mouse models have we seen any signs of toxicity that indicate the production of a toxin by *A. mucolyticum.*

Despite the interesting involvement of CD3 protein family members and *in vitro* data that seems to show binding of *A. mucolyticum* to murine and human T cells, our *in vivo* studies lead us to conclude that the effects of this commensal upon T cells are not strongly activating or inducing clonal expansion. There could be several confounding factors, discussed below, that may obscure a true bacteria-T cell activating interaction, but we have been unable to resolve these issues yet in our present experimental systems. Thus, we rule out this T cell axis as the main cellular driver of inflammation in mice.

3.4. Pitfalls, weaknesses, and alternative interpretations

As noted at the beginning of the chapter, the search for immunostimulatory commensal antigens is greatly hampered by the “dark” genome space of these uncharacterized strains. This challenge has forced us into a variety of approaches hoping that one avenue would produce a thread we could follow towards identification of a bioactive molecule. Rather, as is the frequent experience of many biological researchers, our initial efforts failed to produce such a trustworthy thread, as did the many pilot experiments that were not

included in this thesis. There are a number of weaknesses and issues that have plagued our studies that are worth discussing briefly, if only to help future scientists learn how to avoid the same pitfalls.

One clear weakness of our studies that true microbiologists likely would not have made is a failure to examine the carbohydrate properties of *A. mucolyticum* cell wall components. It is well known that PAMPs like LPS, LTA, MDP, and other building blocks of peptidoglycan activate innate immune receptors and lead to a cascade of signaling events and cellular processes. In our ignorance to examine this basic biochemical fraction, we may have missed the key molecular component of this commensal that mediates its notable effects *in vivo*. Related to this point, in an inverse way, is the possibility that *A. mucolyticum* expresses a protein like a lectin that allows for a broad carbohydrate binding modality, perhaps to glycans that decorate glycosylated proteins. In this way, one bacterial protein would allow for binding of a wide range of host molecules, as seen in our exoproteome screen (Figure 16), that circumvents canonical receptor-ligand binding.

Alternatively, *A. mucolyticum* may express one or more T-independent type 2 (TI-2) B cell antigens, which are known to originate in long, repetitive molecules like pneumococcal polysaccharide and vesicular stomatitis virus glycoprotein serotype Indiana (VSV-G IND) (Bachmann et al., 1993; Dintzis et al., 1983; van Dam et al., 1990). Since we have not yet characterized the nature of *A.*

mucolyticum cell wall components nor their activity on host epithelial or immune cells, it's entirely possible that the observed host activation in this study is primarily driven by B cells. We may also discover this scenario in studies of *A. mucolyticum*-colonized T cell-deficient mice, which are ongoing.

Another weakness in our immunological technical approaches is the possibility that early life thymic selection of T cell precursors may dictate adult T cell reactivity to commensal antigens. All of our gnotobiotic studies were conducted by gavaging adolescent or adult germ-free mice with bacterial inocula well after weaning. Studies of central tolerance and thymic selection have shown that ectopic expression of peripheral antigens by medullary thymic epithelial cells (mTECs) is essential for proper selection of immature thymocytes and formation of a TCR repertoire that is properly suited to its environment (Heino et al., 1999). Were we to breed mice within the MC+Allo microbiota condition, we speculate that this *in utero* and early life exposure to *A. mucolyticum* antigens might change the landscape of T cell reactivity to this commensal strain and allow proper examination of T cell-dependent colitis models. This approach could open up many new fascinating lines of investigation about microbiota influence on maternal-fetal interface, central tolerance, and even autoimmunity, although each is far beyond the scope of the present study.

3.5. Potential solutions and future directions

In order to address the lectin hypothesis, that *A. mucolyticum* is binding many host glycoproteins in a ligand-independent manner, we intend to conduct studies that make use of altered glycosylation patterns. By using enzymes that alter glycosylation or producing recombinant proteins in cell lines with altered glycosyltransferase machinery, we can evaluate *A. mucolyticum* capacity to bind these altered proteins. Taking a different approach, we could search through the *A. mucolyticum* genome for homologs of known lectin proteins, but we've already discussed at length the problems of relying on homology-dependent bioinformatics within the novel commensal microbiome space. Characterization of *A. mucolyticum* cell wall components may instead yield more fruitful answers, but only the data will tell.

Analogous to our ongoing efforts to examine epithelial cell responses to *A. mucolyticum* in vivo (discussed in section 2.5), we similarly wish to examine if murine immune cells exhibit any subtle shifts in their transcriptomes in our scRNAseq dataset. Even if there are not pronounced T cell clonotypes that expand in response to *A. mucolyticum* (perhaps due to the problem of thymic selection, as discussed above), we are intrigued at the possibility that other types of reprogramming, such as stress responses, metabolic shifts, or chromatin remodeling might underlie *A. mucolyticum*-induced immunity. We have yet to examine any of these hypotheses yet in our single cell data but are digging into these reanalyses at the time of this writing.

In silico analysis of *A. mucolyticum* immunogenicity using the BOTA algorithm yielded a number of simple peptides that we have not yet pursued, but we plan to follow up imminently on some of these hits in classic lymphocyte restimulation experiments. Using pools of synthesized peptides, we plan to screen for activation (blasting, proliferation, IL-2 production) of naïve T cells purified from MC+Allo-colonized gnotobiotic mice co-cultured with irradiated antigen presenting cells. In this way we can get a glimpse of the functional performance of these peptides and perhaps hone in on a T cell epitope from this strange CD3-binding commensal strain. Whether or not the true basis of *A. mucolyticum*-mediated immune activation lies in T cell activation, about which we have already expressed our doubts, we seek to define the molecular antigens that lead this strain to generate potent cellular and humoral immune responses in order to develop of future tools that can be used to dissect host-microbe interactions in greater detail.

4. Extended Discussion

4.1. Insights into inflammatory bowel disease etiology

While certain subsets of inflammatory bowel disease (IBD) are known to be driven by genetic susceptibility—for example, mutations in IL10R, NOD2, or ATG16L1—many biomedical researchers have come to appreciate that gut microbiota are involved in disease etiology unilaterally. Although disentangling genetic and environmental components has proven challenging within a wide range of clinical pathologies, the evidence for immunostimulatory gut microbes as aggravators of disease is as strong as ever (Chassaing et al., 2014; Elinav et al., 2011; Franzosa et al., 2019; Garrett et al., 2010; Jostins et al., 2012; Lloyd-Price et al., 2019; Palm et al., 2014). While genetic alleles conferring disease risk are heritable from both father and mother, we note that the heritability of maternal gut microbes remains an equally strong influence on heritability of disease. Decades of therapeutic efforts directed at human targets have left the community with only marginal clinical successes, but the last ten or so years has seen the advent of a microbiome boom. Fecal microbiota transplantation (FMT), probiotics and live biotherapeutics (LBTs) are all surging into the biomedical industry as researchers and clinicians alike seek complementary approaches for disease management.

Our characterization of novel gut commensal taxa within genus *Allobaculum* seeks to provide insight into the environmental side of disease risk and prompt questions for further investigation. Are human subjects colonized with *Allobaculum sps.* more likely to progress to active IBD than those who are not colonized? Within *Allobaculum*-colonized subjects, why do some remain healthy while others develop severe immunopathology? Even though the studies presented herein are not truly preclinical, we suggest that *Allobaculum* strains might be worth investigating as potential therapeutic targets. One recent notable study showed clear enrichment of isolates from the same family as *Allobaculum*, family *Erysipelotrichaceae*, in intestinal creeping fat biopsies from Crohn's disease patients, directly implicating this broader taxon in human IBD (Ha et al., 2020).

Additionally, because of the chronic autoimmune nature of human IBD, we suspect that truly promising candidate microbes will have strong effects on adaptive lymphocytes. Immune activation by commensals, for example pronounced IgA coating in human stool, is a strong predictor of disease involvement, wherein B cells and cognate T cells collaborate in antigenic-specific germinal center reactions to produce high-affinity antibodies against the insulting organism. Analysis of human mucosal plasma cells and lymphocytes demonstrate the involvement of T cells in this process of hypermutated IgA production (Lindner et al., 2012). While our studies offer some conflicting results about the interactions of *A. mucolyticum* and murine T cells (especially in T cell

transfer colitis), we suspect that further work with human samples might reveal a situation more truly reflective of autoimmune IBD driven by bacterial antigens. We also note the value of properly developed chronic models that incorporate early life thymic selection, and urge other researchers to think deeply about such experimental design before making overreaching conclusions based on artificial or inaccurate models.

In order to approach this commensal taxon as a therapeutic challenge, our results suggest that rather than using antimicrobial drugs to try to eliminate *Allobaculum* strains, perhaps leveraging natural microbial interactions will effectively tip the balance of a subject's microbiota in favor of immunoregulation over inflammation. The underlying goal is similar to that of probiotic supplementation, but our approaches are naturally guided and seek to learn from naturally acquired human microbiota rather than exogenous "bugs as drugs".

4.2. Harnessing the complexity of human gut microbiome

As the field continues to innovate and develop new technologies, researchers seek to approximate the full complexity of the human gut microbiome in the laboratory with greater control. One approach that has gained some headway is building large defined communities by culturing and pooling hundreds of strains, which then allows for mechanistic dissection of microbial epistatic relationships at a scale close to that of their natural ecosystem (Cheng et al., 2021). At the

present moment, however, such a model is available to only a few labs in the world.

In our studies of the last five years, we have only made limited forays into experiments near this level of microbiome complexity. Rather, more work is required in this regard and examination of many more complex samples, whether derived from human stool or combinatorial pools of cultured microbes. Our relatively small-scale screen of 19 xenografted human samples revealed *A. muciniphila* as the lead hit (Figure 9C), but a more extensive screen would likely reveal other microbial relationships that are relevant to the human gut. These efforts are necessary for discovery and exploration of microbial pathways that are widely operational in human gut microbiomes. In order to establish the precedent of microbiome studies that move beyond observation into functional mechanism, there is a dire need for harnessing this microbial complexity in a controlled fashion that still eludes the field at large.

There are several avenues by which this lack of complexity in basic research has had major repercussions on the development of microbiota-based tools and therapeutics. First, live biotherapeutics (LBTs) have yet to demonstrate major breakthroughs in efficacy in clinical research because of the highly variable context into which they are delivered in patients' guts. If development of these LBT products were to include vetting in complex microbiota from the ground up, they might not experience the same degree of difficulty in the later stages of

development and implementation in clinics. Second, as the practice of precision or personalized medicine becomes more a reality, clinicians face difficulties with microbiome-based predictive measures and patient stratification. If strain carriage alone is an oversimplified binary criterion for patient inclusion or exclusion, there is an imminent need for more sophisticated models and analyses that incorporate many different microbial strains and interactions (Pacheco and Segre, 2019). More than simple microbiome diversity metrics, great strides are being made in developing new computational approaches for the integration of large-scale multi-omics data in the clinical setting (Lloyd-Price et al., 2019), yet the fundamental mechanistic principles that inform these analytical methods are still being described concurrently. Only by revamping microbiome research at the level of basic science will we see major advances in impact in therapeutic and clinical settings.

Akkermansia muciniphila has recently become one of the most widely-touted gut commensal microbes for its anti-inflammatory and pro-catabolic effects on mammalian hosts (Derrien et al., 2004; Everard et al., 2013; Plovier et al., 2017). Further, a number of studies implicating the microbiome generally, and *A. muciniphila* specifically, in augmenting anti-melanoma therapeutics have attracted the attention of immunologists and oncologists far and wide (Iida et al., 2013; Routy et al., 2018; Sivan et al., 2015; Tanoue et al., 2019). The breadth of literature on the immunostimulatory capacities of *A. muciniphila* is growing and understandably conflicting at times. Notably, this taxon is extremely prevalent in

the general human population, highly IgA-coated, and significantly reduced among IBD patient cohorts (Bajer et al., 2017; Png et al., 2010). However, in certain models *A. muciniphila* appears to exacerbate inflammation and worsen phenotypic outcomes (Cekanaviciute et al., 2017; Ganesh et al., 2013; Khan et al., 2020). Meanwhile, while the prevalence of *Allobaculum* is fairly low (detectable in <10% of healthy volunteer subjects), its effects seem to be profoundly proinflammatory (Miyauchi et al., 2020). In order to disentangle the effects of these two immunostimulatory strains in the human gut, there will need to be substantial further efforts to dissect their ecological interactions and vet their therapeutic efficacy in complex microbial communities.

4.3. Broader effects of microbial epistasis

The reciprocal epistatic interaction we have uncovered between *A. mucolyticum* and *A. muciniphila* is not the first of its kind (Gould et al., 2018; Lengfelder et al., 2019). Yet, these strains' prevalence in humans and potency of the phenotypes observed elevate this paradigm to a level of consideration that is essential for future studies of human-relevant microbes. We predict that epistasis will dictate functional axes of other immunostimulatory microbes in IBD and autoimmunity, and also that microbial epistatic effects will range far and wide beyond immunological activity to influence neurological, metabolic, developmental, and further biological activities.

Just like the functionality of the immune system, we posit that microbiome functionality has co-evolved to a state within mammals that is both broadly active across many body systems and balanced to maintain ecological stability. Put another way, evolution of host-adapted microbes has likely led to extensive diversification such that all available metabolic and functional niches have been filled. Because these niche spaces and intimate host interactions have occurred in mammals over millions of years, it is highly likely that these microbes have co-opted means of communication and modulation of host signals that are specifically operational within those respective niches. We have certainly not described or observed many of these functional niches yet, nor identified the specific microbes that fill them, but, as a society, we have finally begun to appreciate the powerful influence these microorganisms exert on many aspects of our basic biology.

Aside from the specific molecular mechanisms that remain to be discovered, an important feature of the microbiome that our manuscript has not directly addressed is that of colonization resistance. We admit that there is a possibility that some data we have presented might be explained by a generic feature shared across many different microbial taxa, wherein strain A precludes the colonization of strain B not by a specific molecular modulation, but by simply excluding strain B from gaining a colonizing foothold, typically by outcompeting for nutrients. While our study hasn't formally addressed this question by performing sequential colonizations with our strains of interest, we note that our

phenotypic results have displayed strong penetrance despite quantitative fluctuations in microbial abundance from mouse to mouse. If there is a key site where CFU burden dictates outcome to a very sensitive degree, we have not yet found this inductive site, but acknowledge that it could hypothetically exist. For example, *Alcaligenes sp.* is reported to inhabit the murine Peyer's patches (PP), which is clearly a privileged site wherein this bacterial strain might dramatically influence host responses simply by its persistence (Obata et al., 2010). We grant that while this type of work is technically challenging, it will likely be very fruitful in revealing the finer aspects of colonization resistance that nearly all humans benefit from.

4.4. Molecular communication between host and microbe

One of the open questions that looms large in the field is the role of "common chemical currency" by which microbes and hosts communicate at the molecular level. Small molecule metabolites that are biotransformed by gut microbes unarguably have a profound effect on host physiology, allowing for communication via the same molecular "language", but more time and broader understanding are necessary to slowly reshape the immunology community's understanding of microbial detection. Many immunologists, the authors included, tend to think of protein and carbohydrate antigens derived from commensal and pathogenic microbes as the most important forms of microbial information received by the mammalian immune system. In reality, there are likely so many

other biological signals which are not recognized by recombined antigen receptors that we may need to recategorize our understanding of commensal-derived molecules altogether. Advances in metabolomics have unveiled a whole world of chemical communication, and characterization of GPCR and other host receptor activation by microbial metabolites have begun to “decode” this chemical communication between host and microbes (Chen et al., 2019; Guo et al., 2019). Pathogen-associated molecular pattern (PAMP)-based activation of pattern recognition receptors (PRRs) was one concept that attempted to describe these features and activities beyond the scope of antigen receptors, but perhaps even further paradigms need to be proposed to encompass these chemical communication modalities.

Even so, classical protein antigens are indeed influential and provide clues about immunological recognition of key microbial molecules. Even though our studies of *A. mucolyticum* immunogenicity have come up “dry” in that sense, we suspect there will be an identifiable molecular epitope to find in the coming years of research. We find this question to be worthy of pursuit, not only because of its place in the immunostimulatory human microbiota, but also because it may come to offer utility for future researchers in a yet-unknown way, such as a model antigen or a technological tool. The recent applications of CRISPR-Cas systems in genome editing is proof enough that naturally occurring systems can have incredible utility when understood deeply.

Finally, this work revealed to us that, aside from molecules themselves, *behaviors and locations* of commensal bacteria may also serve as activating signals in a way that is conserved across taxa. For example, SFB attachment to epithelial cells activates endocytosis of microbial P3340 in a contact-dependent fashion, leading to transcription of high levels of Saa1 and Duox2, which is a common response to other adhesive bacteria (Atarashi et al., 2015; Ladinsky et al., 2019). As mentioned above, *Alcaligenes* persistence in PPs leads to IgA induction (Obata et al., 2010), and further evidence of commensal inhabitation of privileged immune inductive sites may soon emerge. We have yet to identify whether the key aspect of *Allobaculum mucolyticum* host activation is a protein antigen, a small molecule, or a biogeographical location, but find it a remarkable member of the microbiome nonetheless, and continue to strive to understand it more deeply.

In summary, we have shown that novel human commensal isolates belonging to genus *Allobaculum* are immunostimulatory *in vivo*, and discovered that this activity depends on a reciprocal epistatic interaction with commensal *Akkermansia muciniphila.* Not only are these bacterial taxa implicated in gut microbiota homeostasis and inflammatory disease states, but their interaction also points to the likely existence of other similar epistatic axes between other commensal microbes and far-reaching effects upon mammalian biology.

5. Materials and Methods

5.1 Key Resources Table

Bacterial Strains					
Strain designation	**Taxonomic assignment**	**Isolation reference**	**Culture Media**	**Growth temp**	**Growth Atmosphere**
NWP_0582	*Bacteroides sp.*	Palm & de Zoete, et al. Cell 2014.	GMM (Goodman et al., 2011)	37˚C	Anaerobic
NWP_0583	*Parabacteroides distasonis*	Palm & de Zoete, et al. Cell 2014.	GMM	37˚C	Anaerobic
NWP_0584	*Peptoniphilus sp.*	Palm & de Zoete, et al. Cell 2014.	GMM	37˚C	Anaerobic
NWP_0585	*Bacteroides ovatus*	Palm & de Zoete, et al. Cell 2014.	GMM	37˚C	Anaerobic
NWP_0586	order Clostridiales UC	Palm & de Zoete, et al. Cell 2014.	GMM	37˚C	Anaerobic
NWP_0587	family Lachnospiraceae UC	Palm & de Zoete, et al. Cell 2014.	GMM	37˚C	Anaerobic

NWP_0588	*Collinsella stercoris*	Palm & de Zoete, et al. Cell 2014.	GMM	37˚C	Anaerobic
NWP_0589	*Bacteroides uniformis*	Palm & de Zoete, et al. Cell 2014.	GMM	37˚C	Anaerobic
NWP_0590	*Parabacteroides sp.*	Palm & de Zoete, et al. Cell 2014.	GMM	37˚C	Anaerobic
NWP_0324	*Allobaculum sp.* 128	Palm & de Zoete, et al. Cell 2014.	Gifu	37˚C	Anaerobic
NWP_0593	*Allobaculum sp.* ‘Allo2’	this paper	Gifu	37˚C	Anaerobic
ATCC BAA-835	*Akkermansia muciniphila*	Derrien et al., 2004	Gifu	37˚C	Anaerobic
NWP_0598	*Akkermansia muciniphila* ‘2G4’	this paper	Gifu	37˚C	Anaerobic
Culture Media					
Gifu Anaerobic Media		HyServe	05422		
Gut Microbiota Media (GMM)		Goodman, et al. 2011			
Mouse strains					
Germ-free C57BL/6	University of Chicago Animal Resources Center				

Germ-free *IL10*$^{-/-}$	University of Michigan Gnotobiotics	https://microbe.med.umich.edu/services/germ-free-gnotobiotic-mouse-facilities			
Germ-free *Rag1*$^{-/-}$	University of Michigan Gnotobiotics	https://microbe.med.umich.edu/services/germ-free-gnotobiotic-mouse-facilities			
Antibodies and Staining Reagents					
Vendor	**Catalog #**	**Product**	**Clone**	**Conj.**	**Working concentr.**
BD	551460 (Lot 6033810)	Rat anti-Ms Ly6G	1A8	FITC	2.5 µg/mL
BD	553729 (Lot 5191688)	Rat anti-Ms CD4	GK1.5	FITC	1 µg/mL
BD	612821 (Lot 9331214)	Hamster anti-Ms TCRbeta	H57-597	BUV737	1 µg/mL
BD	557659 (Lot 5357842)	Rat anti-Ms CD45	30-F11	APC-Cy7	1 µg/mL
BD	558214 (Lot 5009871)	CD3e	500A2	Pacific Blue	1 µg/mL
BD	560184 (Lot 7104724)	IL-17A	TC11-18H10	AF647	2 µg/mL
BD	563413 (Lot 5170799)	MHCII	M5/114.15.2	BV605	1 µg/mL
BD	565976	CD11b	M1/70	BUV395	1 µg/mL
BioLegend	109807 (Lot B209477)	Anti-Ms CD45.2	104	PE	0.5 µg/mL

BioLegend	405322 (Lot B246161)	Goat anti-Ms IgG	poly4053	AF647	1.2 µg/mL
BioLegend	103255 (Lot B223589)	B220	RA3-6B2	BV711	0.5 µg/mL
BioLegend	128015	Ly6C	HK1.4	APC	1 µg/mL
eBioscience / Thermo Fisher Scientific	12420482 (Lot 2173312)	Rat anti-Mouse IgA	mA-6E1	PE	4 µg/mL
eBioscience / Thermo Fisher Scientific	14016186 (Lot 4333612)	Anti-Ms CD16/32 “FcBlock”	93	n/a	1 µg/mL
eBioscience / Thermo Fisher Scientific	56011480 (Lot E089601632)	CD11c	N418	AF700	1 µg/mL
Invitrogen / Thermo Fisher Scientific	31430 (Lot UB278606)	Gt anti-Ms IgG (H+L)	poly	HRP	1:6,000 (0.13µg/mL)
Sigma Alrich	A4789	Gt anti-Ms IgA	poly	HRP	1:6,000
Southern Biotech	156011 (Lot G3512PD25W)	Rat anti-Ms CD11b	3A33	APC	1 µg/mL
Thermo Fisher Scientific	L23105	Live/Dead Fixable Blue	n/a	Ex/Em 350/ 450nm	1:1,000
Critical Commercial Assays					
MagAttract Microbial DNA Kit (384)		Qiagen	27200-4		

PCR Purification Kit, Agencourt AMPure XP	Beckman Coulter	A63881
NGS Library Quantification Complete kit (ABI Prism)	KAPA Biosystems	KK4835 (Roche 07960204001)
MiSeq Reagent Kit v2 (500 cycles)	Illumina	MS-102-2003
Mouse Lipocalin-2/NGAL DuoSet ELISA	R&D Systems	DY1857
Other Reagents		
Dextran Sodium Sulfate (DSS)	TdB Labs	https://shop.tdblabs.se/products/dextran-sulfates-sodium
Bouin's Fixative Solution	Fisher Scientific	11201
TRI reagent	Sigma Aldrich	T9424
Bioanalyzer RNA 6000 Nano Kit	Agilent	5067-1511
TMB Substrate Kit	Thermo Fisher Scientific	34021
Sulfuric Acid, 2.0 Normal	Avantor	H381-05
RPMI 1640	Thermo Fisher Scientific	11875-119
DNase I	Sigma Aldrich	10104159001
Collagenase D (type IV)	Sigma Aldrich	11088882001 (Roche COLLD-RO)

Percoll	VWR	89428-524
PMA (Phorbol-12-myristate-13-acetate)	Sigma Aldrich	P1585-1MG
Ionomycin Calcium Salt, Ready Made	Sigma Aldrich	I3909-1ML
GolgiStop Protein Transport Inhibitor	BD	554724
Equipment		
Anaerobic Culture Chambers	Coy	Custom-built
Flexible Film Gnotobiotic Isolators	Class Biologically Clean	n/a
Isocage P Microisolator Caging System	Techniplast	ISO72P
Bead Beater	Biospec	https://biospec.com/instructions/bead beater
Spectramax i3x plate reader	Molecular Devices	i3x
QuantStudio 6 Flex Real-Time PCR instrument	Applied Biosystems	4485699
Services		
Wet-to-Digital H&E histology	Histowiz	https://home.histowiz.com/
Software and Algorithms		
GraphPad Prism v9	https://www.graphpad.com/scientific-software/prism/	
QIIME v1.9	http://qiime.org/	

MEGA v10.2.6	https://www.megasoftware.net/
FlowJo v10	Treestar
Partek Flow v6	https://www.partek.com/partek-flow/
Panther v14	http://geneontology.org
Seurat v3.2.1	https://satijalab.org/seurat/index.html
Immunarch v0.6.6	https://immunarch.com/
R v4.0.3	https://www.r-project.org/

5.2 Resource Availability

Materials availability. Further information and requests for resources and reagents should be directed to the Lead Contact, Noah W. Palm (noah.palm@yale.edu).

Data and code availability. Whole genome sequences (GenBank accessions CP078088 and CP078089), fecal 16S microbiota profiles, and bulk colon RNAseq data are available at SRA Bioproject PRJNA739762. Single cell RNAseq data are available at GEO GSE179165.

5.3 Experimental Model and Subject Details

Bacterial Strains. Frozen stocks of each strain were streaked on Gut Microbiota Media agar (Goodman, et al. 2011) or Gifu Anaerobic Media agar (HyServe

#05422) and incubated 48h at 37˚C. Unless otherwise noted, all bacteria were grown in anaerobic conditions (gas composition: 4% H2, 10% CO2, 86% N2). Single colonies were picked into sterile broth and grown overnight at 37˚C without shaking. 10µl aliquots of overnight broths were removed for alkaline lysis with boiling to extract genomic DNA, then identities of these monocultures were confirmed by PCR amplification of the 16S rRNA gene V4 region and Sanger sequencing (V4_F: GTGCCAGCMGCCGCGGTAA, V4_R: GGACTACHVGGGTWTCTAAT) (or full length 16S rRNA gene, using published primer sequences 8F and 1391R). Sequences were queried against NCBI and RDP databases.

Human fecal samples. Human study protocols were approved by the Institutional Review Board (HIC # 1607018104) of Yale School of Medicine. Informed consent was obtained from all participants and/or their legal guardians and all methods were performed according to relevant guidelines and regulations. Healthy subjects were recruited via advertisements on the Yale medical campus and in the New Haven Public Library. All fecal samples in this study were collected at home and stored on ice packs at −20 °C before either overnight shipment or direct laboratory drop-off the day following collection in an insulated container. Samples were then stored at -80 °C until use.

Animal experiments. Germ-free mice (BL/6, RAG1-/-, IL10-/-) were maintained in flexible film isolators (CBC) with all bedding, chow (Teklad 2018S), and water

being autoclaved before import. All germ-free breeding isolators were regularly monitored for the presence of bacteria (both culture-dependent and -independent techniques). All experiments were conducted by transferring mice to positive pressure ventilated microisolator cages (Techniplast #ISO72P), and inoculating each mouse by oral gavage immediately upon transfer. Inocula were previously prepared in anaerobic culture and frozen at -80˚C in media + 20% glycerol in gasket-sealed airtight glass vials (Wheaton). The day of inoculation, Wheaton vials were thawed to 25˚C and 0.1mL gavaged per mouse. All animal protocols were approved by Yale University Institutional Animal Care and Use Committee (IACUC Protocol 2018-11513). All animal experiments were replicated in both male and female mice of 6-8 weeks of age. Dextran Sodium Sulfate (DSS; TdB Labs) was dissolved in sterile H2O to 2% w/v and passed through a 0.2μm vacuum filter before ad libitum administration. Serum was collected under isoflurane anesthesia, by retro-orbital puncture.

5.4 Method Details

Fecal sample processing. Freshly defecated fecal samples were collected into sterile 2mL screw-cap tubes and rehydrated in 1mL sterile PBS, disrupted by 10sec bead beating (Lysing matrix D beads, MP Biomedicals) in a Biospec bead beater, then centrifuged 5min at 50xg to gently pellet large debris. Bacterial cell suspension was then transferred to sterile 2mL deep-well plates for downstream processing. Fecal bacteria were pelleted at 10,000xg for 10min, and clarified fecal water was removed for evaluation of Lipocalin-2 content by ELISA (R&D

Systems DY1857). Bacterial pellet was resuspended in Qiagen PowerBead buffer, sonicated for 5min in sonicating water bath, lysis buffer was added, then complete lysis achieved by 0.1mm bead beating followed by genomic DNA isolation (Qiagen DNeasy Ultraclean Microbial; cat #12224).

Microbiota profiling. The 16S rRNA gene V4 region was amplified from each bacterial gDNA sample by PCR according to a dual-index multiplexing strategy (Kozich et al., 2013), then amplicons were normalized and cleaned (Agencourt AMPure XP purification beads; Beckman Coulter #A63881). Samples were pooled and libraries were quantified by qPCR (KAPA Biosystems KK4835; Applied Biosystems QuantStudio 6 Flex instrument) then sequenced on an Illumina Miseq in (2x250 PE using 500 cycle V2 reagent kit #MS-102-2003).

Whole genome sequencing. Overnight bacterial cultures were harvested by centrifugation, cells were lysed for high molecular weight gDNA extraction (Quick-DNA HMW MagBead Kit; Zymo Research #D6060). Genomic DNA was used to prepare two different types of sequencing libraries. Illumina's Nextera XT kit (#FC-131-1024) was used to prepare short-read libraries, which were sequenced on Illumina Miseq (2x250), while Oxford Nanopore Technologies Ligation Sequencing kit (#SQK-LSK109) was used to prepare long-read libraries, which were sequenced using ONT MinION (Flow cell R9.4.1; #FLO-MIN106D).

Antibiotic susceptibility testing. Overnight bacterial cultures were spread onto Gifu agar plates to form a lawn, then MIC E-test strips (Biomerieux) were overlaid on top of the lawn. Agar plates were incubated at 37˚C for 48h before determination of MIC, where the zone of clearance meets the edge of the test strip.

Histology. Whole mouse colons were placed in plastic histology cassettes and immersed in Bouin's fixative fluid for 24h before transfer to 70% ethanol, paraffin embedding, sectioning, and H&E staining. Blinded slides were scored by a board-certified pathologist.

RNA-seq. Colon tissues were opened longitudinally and washed thoroughly in sterile PBS until no visible fecal debris remained, then finely minced with a razor blade and transferred to 2mL screw-cap tubes with 1mL ice-cold TRI Reagent (Sigma Aldrich #T9424) and nuclease-free 0.1mm glass beads, thoroughly bead beating for 20sec *3, resting on ice in between. Bulk RNA samples were cleaned using Qiagen RNeasy Mini columns, DNase I digested, and quality checked on an Agilent Bioanalyzer RNA 6000 Nano Kit (#5067-1511). Sequencing libraries were prepared by Yale Center for Genome Analysis staff and run using Illumina Hiseq 2x75 chemistry.

Fluorescence in situ hybridization. 1cm segments of mouse tissue were excised and fixed in Carnoy's solution (1 Acetic Acid : 3 Chloroform : 6 Ethanol)

for no more than 2 hours. Fixed tissues were embedded in paraffin for 5µm cryosectioning. Slides were deparaffinized in xylenes, rinsed in ethanol, and dried thoroughly before hybridization. Bacterial probe EUB-338 ([Cy3]-5'–GCTGCCTCCCGTAGGAGT-3'-[Cy3]) was used for staining at 1µg/mL in hybridization buffer (0.9M NaCl + 0.02M Tris, pH 7.5 + 20% Formamide + 0.05% SDS) in a humidified chamber for 2h at 46˚C. After washing, slides were counterstained with DAPI and mounted in ProlongGold Antifade mounting media with overnight curing. Images were acquired on a Leica SP8 confocal microscope running LAS-X software version 3.1.5.

Bacterial flow cytometry. Fecal bacterial cell suspensions were transferred to sterile LB+20% Glycerol and frozen at -80˚C until further analysis. Bacteria were thawed on ice, then aliquoted 10^4 - 10^5 CFU per well of 2mL 96-deep-well plate (pellet not visible) (Moor et al., 2016). Each staining reaction was blocked with normal rat serum for 15min, then washed in sterile PBS/0.1%BSA. Staining for endogenous coating by mouse IgA was performed at 1:100 with PE-conjugated eBioscience clone mA-6E1 (Thermo Fisher #12420482). Cells were washed three times in 500µl PBS, then transferred to 1.1mL microdilution tubes (VWR 20901-013) for analysis on a BD FACS Calibur instrument, including control tubes for sterile buffer (log FSC, log SSC), unstained cells, and secondary only-stained cells to set appropriate gates. A minimum of 50,000 events/sample were collected and analyzed using FlowJo v9.

SDS-PAGE and Immunoprecipitations. Overnight broth cultures were harvested by centrifugation and crude lysates prepared using Triton-X-100 lysis buffer, centrifuged again to separate cell wall debris, and soluble fraction mixed with Laemmli loading buffer with 2-mercaptoethanol, and loaded onto 4-15% Tris-Glycine gels (Bio-rad). After running, gels were either stained with Coomassie Blue or blotted onto nitrocellulose membranes, blocked with 1% BSA, then probed with mouse antiserum overnight, followed by detection antibodies: HRP-conjugated Goat-anti-Mouse IgG (Thermo Fisher Scientific #31430; 1:6,000 dilution). For immunprecipitation studies, bacterial lysates were pre-cleared by incubation with normal mouse serum on a rotator for 30min, then washed over Protein A/G beads 3 times in PBS-Tween, followed by incubation with immune serum from MC+Allo mice of interest on a rotator for 2h, then bound to Protein A/G beads, washed 3 times in PBS-T, then eluted in Glycine pH5 and immediately neutralized with Tris. These eluates were run on SDS-PAGE gels, as above, comparing to input and mouse serum control lanes, for determination of unique bacteria-derived bands of interest. Coomassie-stained bands were excised and submitted to Keck Proteomics Core Laboratory for LC/MS.

Bacterial ELISAs. Overnight broth cultures of bacterial strains of interest were washed three times in sterile PBS, then normalized to an OD600 of 0.1. Many 100µl aliquots were prepared and snap frozen in liquid nitrogen. To prepare ELISA plates, bacterial aliquots were thawed on ice, diluted further 1:10 in PBS, then coated 50µl/well of Nunc Maxisorp Immunoplates overnight at 4˚C. The next

day plates were spun 15min at 5000xg before discarding supernatant and confirming bacterial adhesion by phase contrast microscopy. Plates were blocked with 1%BSA in PBS before serially diluting serum or fecal water. After 2h incubation at RT, plates were washed four times with TBS-T, then mouse IgG was detected using HRP-conj. Goat anti-Ms-IgG (Thermo Fisher Scientific #31430; 1:6,000 dilution), or mouse IgA using HRP-conj. Goat anti-Ms-IgA (Sigma Aldrich A4789; 1:6,000 dilution). Plates were washed four times before detection with TMB (Pierce), stopped with 2N H_2SO_4, and read at Abs 450nm (Molecular Devices SpectraMax i3x).

Colon lamina propria cell isolation. Colon tissue was harvested into 25˚C complete RPMI 1640 medium (supplemented with 10% FBS, Pen-Strep, L-Glutamine, HEPES). After gentle cleaning to remove large fecal debris, tissues were shaken in HBSS + 1.5mM EDTA at 37˚C 225rpm for 20min x2 to remove mucus and epithelial layers. Then lamina propria tissue was minced and transferred to cRPMI + 0.5mg/mL DNase + 1mg/mL Collagenase D for 45min at the same speed. Then cells were filtered twice through stainless steel mesh and lymphocytes enriched in a 40%-70% Percoll interface (20min at 600xg, brake off). Then cells were aliquoted to round-bottom polystyrene microplates for Fc Blocking, fluor-conjugated antibody staining, and washing. Ex vivo cell restimulations were performed with 50ng/mL PMA + 1μM ionomycin, in the presence of brefeldin A (GolgiStop reagent, BD #554724), before surface staining, fixation, permeabilization, and intracellular staining.

MLN & PP cell isolation. Mucosal lymphoid tissues were dissected and gently washed in sterile PBS, transferred to digestion media (serum-free RPMI 1640 supplemented with, Pen-Strep, L-Glutamine, HEPES, 2-mercaptoethanol, NEAA, Sodium Pyruvate, DNase I, and Collagenase D) in 30mL beaker with a small magnetic stir bar and stirred at 400rpm in 5%CO2 incubator for 15min. After stirring, beakers were transferred to ice and triturated with media containing 3% FBS, filtered through stainless steel mesh, centrifuged 350xg 10min 4˚C. Cells were washed twice more in media to remove large debris chunks, then resuspended in PBS + 0.04%BSA and filtered again through 40μm nylon.

Single-cell RNA sequencing. Single cell suspensions were counted by hemacytometer and normalized to 1e6/mL for submission to Yale Center for Genome Analysis staff for droplet generation and gel bead encapsulation using 10X Genomics Controller. Cell lysis, barcoding, and reverse transcription were performed using Chromium 5' V2 chemistry according to manufacturer's instructions. PCR-amplified gene expression libraries were quantified and evaluated for QC by Agilent Bioanalyzer, and sequenced on Illumina NovaSeq 6000 at a depth of 175M read pairs per library.

Bioinformatic analyses. Phylogenetic analysis of bacterial taxa belonging to family *Erysipelotrichaceae*: 16S rRNA gene sequences from NCBI Genbank were aligned using Clustal Omega and alignments imported into MEGA v10.2.6.

Phylogenetic trees were constructed using both neighbor-joining method and maximum likelihood estimation method, in each case bootstrapping for 1,000 replicates, both of which resulted in the same overall phylogeny. Trees were visualized using interactive Tree of Life (Letunic and Bork 2021). Whole genome assemblies: long-read fastq files were passed to Flye v2.6 for assembly (Kolmogorov et al., 2019) and short-read fastq files were used to finish assembling remaining contigs using Unicycler v0.4.9b (Wick et al., 2017). Microbiota profiling: 16S rRNA amplicon sequencing data were processed and analyzed using QIIME (v1.9), including rarefaction to 1000 reads/sample, elimination of reads below a frequency of 0.0001, open reference OTU picking, and filtering out contaminating OTUs known to originate from water control PCRs (Caporaso et al., 2010; Lozupone et al., 2012; McDonald et al., 2012). Bulk RNAseq sequencing data were trimmed, aligned, and gene counts quantified using Partek Flow (v6.0). Gene lists were analyzed for GO enrichment using Panther v14 available at http://geneontology.org (Mi et al., 2019). Single cell sequencing data were demultiplexed then processed using 10X Genomics cellranger count. Count matrices were imported into Seurat (v3.2.1) within R (v4.0.3) (Butler et al., 2018), paired with microbiome metadata, filtered for nFeature_RNAs <500 & <6000 and percent mitochondrial genes <8%. Clusters were generated by UMAP with resolution = 0.8, manually annotated based on expression of conserved marker genes, then analyzed for differential expression across microbiome groups using FindMarkers. TCR repertoires were analyzed using Immunarch (Immunomind Team 2019).

Quantification and Statistical Analysis. Statistical analysis was conducted in GraphPad Prism v9. Unless otherwise noted, data are plotted as mean ± SEM. Each figure legend describes the sample sizes of the data shown in that figure, as well as the specific statistical tests applied.

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Appendix 1. Supplementary Figures.

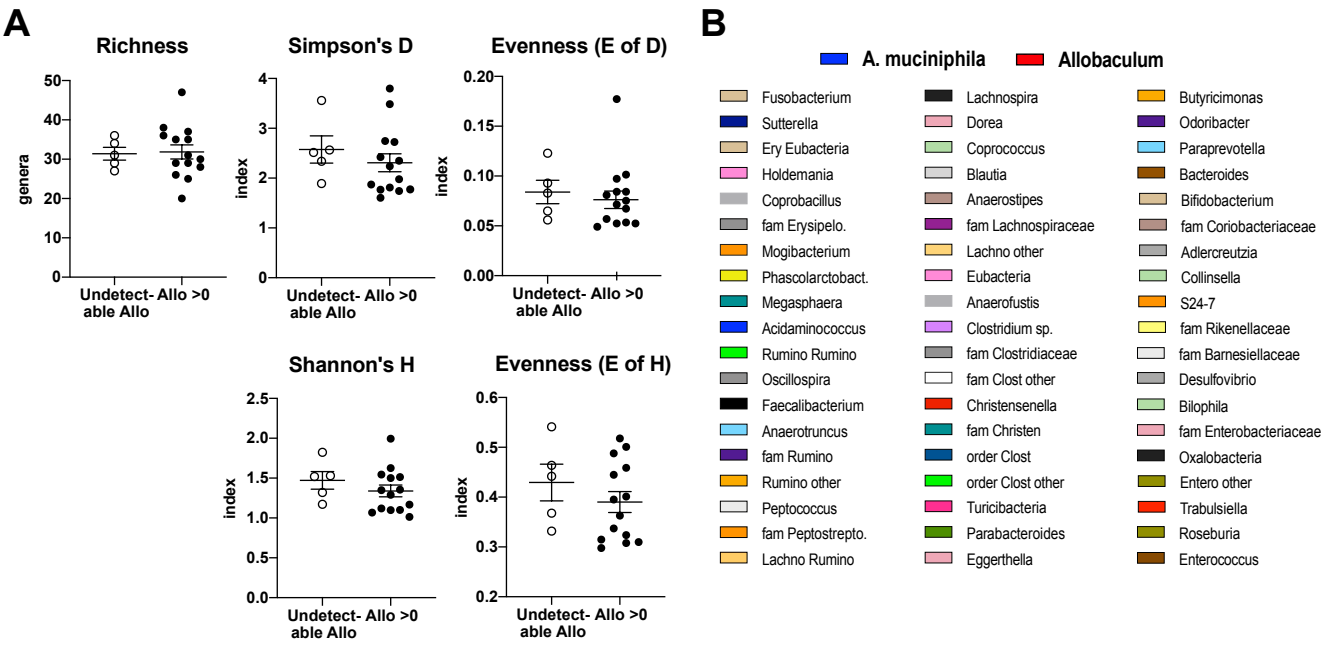

Figure S1. Microbial ecological diversity fails to explain *A. mucolyticum* gut persistence in transplanted healthy human microbiota. (A) Genus-level richness, Simpson's diversity index, Shannon's diversity index, and evenness of each microbiome that contained *A. mucolyticum* (n=14) or lacked *A. mucolyticum* (n=5). (B) Legend accompanying Figure 9B.

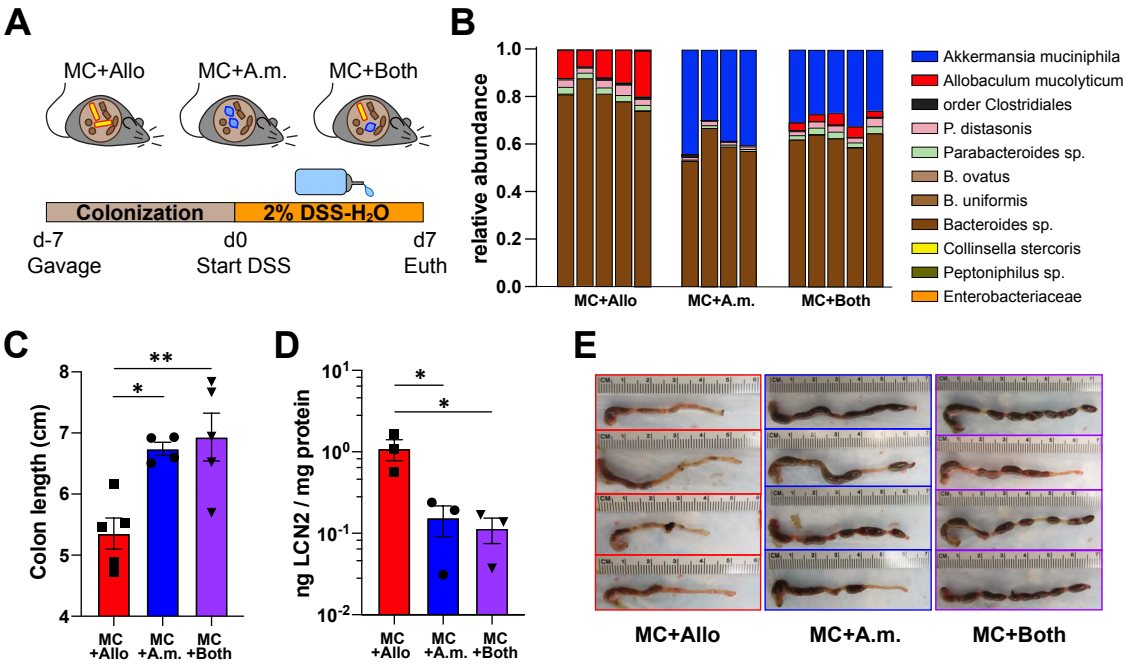

Figure S2. Second *A. muciniphila* isolate attenuates *A. mucolyticum*-mediated colitis. (Accompanies Figure 10) (A) Experimental schematic for acute DSS colitis in WT gnotobiotic mice colonized with MC+*A. mucolyticum*, MC+*A. muciniphila* (in-house isolate 2G4), or MC+*A.mucolyticum*+*A. muciniphila* (2G4)(n=4-6 mice/group). (B) Fecal microbiota profiling, (C) Colon length, (D) d2 fecal lipocalin (LCN2), and (E) gross colon pathology. Welch's t-test was used to compare microbiota groups. *P<0.05.

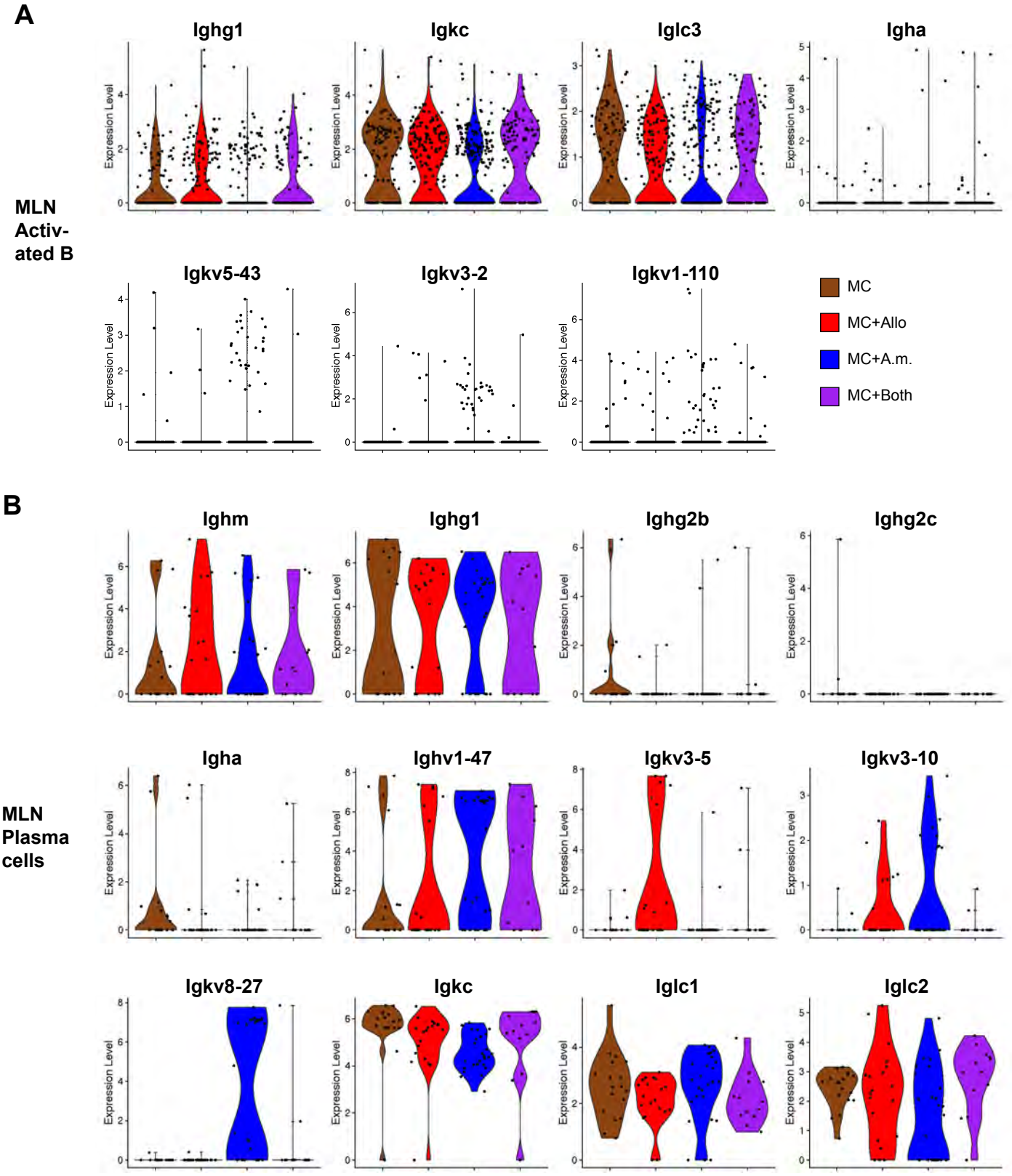

Figure S3. Expression of heavy and light chain Immunoglobulin (Ig) genes in MLN B-lineage cells. Most differentially expressed Ig genes across microbiome groups in (A) Activated B cells and (B) Plasma cells. The remaining immature B cell clusters expressed Ighm nearly exclusively.

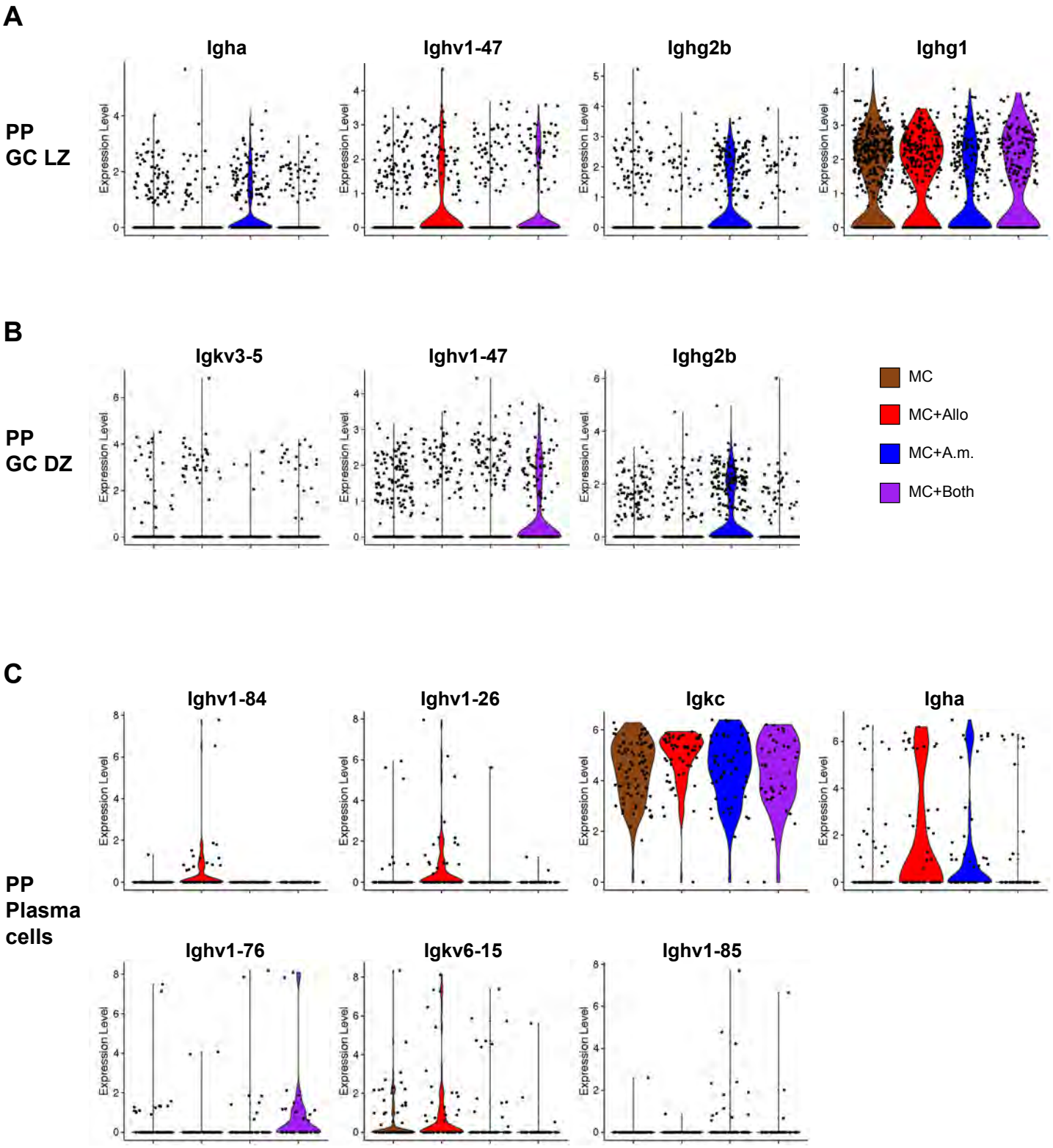

Figure S4. Expression of heavy and light chain Ig genes in PP B-lineage cells. Most differentially expressed Ig genes across microbiome groups in (A) Germinal center light zone B cells (GC LZ), (B) Germinal center dark zone B cells (GC DZ), and (C) Plasma cells. The remaining immature B cell clusters expressed Ighm nearly exclusively.