Thesis

BACTERIAL MEMBRANE VESICLES MEDIATE INTESTINAL HOMEOSTASIS

Exploring the Impact of *Akkermansia muciniphila*-derived Membrane Vesicles on the Mucus Barrier

submitted by

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2 Zusammenfassung

Einleitung: Die Aufrechterhaltung der Symbiose aus Mikrobiom und Darm erfordert ein fein abgestimmtes Gleichgewicht aus Pathogeneliminierung und Toleranz gegenüber kommensaler Bakterien. *Akkermansia muciniphila* ist einer dieser wichtigen Kommensalen und mit der Verstärkung der Epithel- und Mukusbarriere sowie Immunmodulation assoziiert. Wie der Name verspricht, ist *A. muciniphila* auf das Mukus-Habitat des Darms spezialisiert. Eine Reduktion *von A. muciniphila* im Mikrobiom steht in Verbindung mit vielen Krankheiten und einer dysfunktionalen Mukusbarriere. Bakterielle Membranvesikel (BMVs) ermöglichen die Kommunikation zwischen Wirt und Bakterien, indem sie die Mukusbarriere durchqueren. Mittlerweile ist bekannt, dass *A. muciniphila* viele der unterstützenden Effekte für den Wirt durch seine Outer Membrane Vesicles (OMVs) vermittelt. Sie verringern die Permeabilität der epithelialen Barriere, modulieren das Immunsystem und unterstützen hierdurch die Besiedlung durch Kommensalen. BMVs spielen eine essenzielle Rolle in der Aufrechterhaltung der intestinalen Homöostase. Bislang wurde jedoch nicht untersucht, ob *A. muciniphila*'s OMVs auch dessen Einfluss auf das Mukussystem mediieren. Dieses Wissen könnte wichtige Erkenntnisse für das Potenzial von BMV-basierten Therapien bei chronisch entzündlichen Darmerkrankungen (CED) liefern. Diese Krankheiten sind durch eine reduzierte Präsenz von *A. muciniphila* sowie eine gestörte Mukusbarriere gekennzeichnet.

Methoden: Zur Untersuchung der Effekte von *A. muciniphila* OMVs auf das Mukussystem, konzentrierten wir uns auf Parameter der Becherzelldifferenzierung und die MUC2 Expression in intestinalen Zelllinien. Die MUC2 Expression lässt nicht nur Aussagen über die Mucinproduktion zu, sondern dient auch als Marker für Becherzellen. So wurde mithilfe der Durchflusszytometrie der Anteil an LS174T-Zellen, die MUC2 exprimieren, bestimmt. Außerdem wurde das intrazelluläre MUC2-Vorkommen quantifiziert. Um weitere mechanistische Einblicke zu erlangen, wurde eine RT-qPCR ergänzt, um die mRNA-Expression von Markern der Becherzelldifferenzierung und verschiedenen Mucinen in HT-29-MTX-Zellen zu ermitteln.

Ergebnisse: Die Durchflusszytometrie ergab, dass LS174T-Zellen im Vergleich zu anderen Darmzelllinien, einschließlich HT-29 und HT-29-MTX, signifikant höhere MUC2-Spiegel aufwiesen. Die Behandlung mit dem γ-Secretase-Inhibitor DAPT induzierte ausschließlich in LS174T-Zellen eine erhöhte MUC2-Expression ($p < 0{,}01$). Die Behandlung mit *A. muciniphila* OMVs zeigte jedoch weder einen signifikanten Einfluss auf die mittlere Fluoreszenzintensität, noch auf den Anteil der MUC2-positiven Zellen. Die RT-qPCR ergab zudem, dass die mRNA-Expression von *MUC2, MUC1, MUC5AC, KLF4 und AGR2* durch die OMV-Behandlung unverändert blieb.

Diskussion: Diese Untersuchung vereinfachte die hochkomplexe in vivo Situation in ein experimentelles Modell, in dem nur ein Bruchteil des Mukussystems analysiert wurde. Daher können mit diesem Ansatz nicht alle potenzielle Auswirkungen von *A. muciniphila* OMVs erfasst werden. Zusätzlich erfordert die experimentelle Verwendung von BMVs weitere Evaluierung und Standardisierung. BMVs bieten dennoch einen interessanten Ansatz zur Wiederherstellung der intestinalen Homöostase und werden damit als neue therapeutische Strategie für CEDs diskutiert. Abschließend wird ein konzeptionelles Modell vorgestellt, das die mögliche Rolle von BMVs in der Pathophysiologie der CEDs veranschaulicht. Hier werden die CEDs als gestörte bidirektionale Kommunikation zwischen Wirt und Mikrobiom illustriert.

3 Abstract

Introduction: Maintaining symbiosis between the gut microbiome and its host requires a delicate balance between pathogen clearance and tolerance towards commensal bacteria. *Akkermansia muciniphila* is a key beneficial commensal that enhances epithelial barrier integrity and mucus production while inducing immunomodulation. As the name suggests, *A. muciniphila* is specialized in the mucosal habitat of the gut. Reductions in its abundance are associated with various diseases and a dysfunctional mucus barrier. Bacterial membrane vesicles (BMV) enable communication between host and microbiota by passing the mucus barrier. *A. muciniphila* mediates many beneficial effects by its outer membrane vesicles (OMV). They reduce intestinal permeability, modulate the immune system and ultimately support colonization with commensals. BMVs seem to play an essential role in maintaining intestinal homeostasis, but it is unknown whether *A. muciniphila*'s OMVs also mediate its effects on the mucus barrier. Exploring this could provide valuable insights for the use of BMVs as a novel therapy option for inflammatory bowel diseases (IBD). These diseases are characterized by reduced *A. muciniphila* abundance and an impaired mucus barrier.

Methods: To investigate the effects of *A. muciniphila* OMVs on the mucus system, we focused on goblet cell differentiation and MUC2 expression in intestinal cell lines. MUC2 expression not only measures mucus production but also suits as a goblet cell marker. Flow cytometry was used to determine the ratio of MUC2-positive LS174T cells and quantify their intracellular MUC2 levels. To gain deeper mechanistic insights, RT-qPCR was employed to assess the mRNA expression of goblet cell differentiation factors and mucins in HT-29-MTX cells.

Results: In flow cytometry, we observed that LS174T cells expressed higher levels of MUC2 compared to other intestinal cell lines like HT-29 and HT-29-MTX. Treatment with the γ-secretase inhibitor DAPT resulted in an upregulation of MUC2 expression only in LS174T cells but not HT-29-MTX cells. However, *A. muciniphila*-OMV treatment did not significantly affect the number of MUC2-

positive cells. RT-qPCR further showed that the expression of *MUC2, MUC1, MUC5AC, KLF4, and AGR2* mRNA remained unaffected following OMV treatment.

Discussion: This study has simplified the highly complex in vivo situation to an experimental model, which analyses only a fraction of the mucus system. As a result, broader effects of *A. muciniphila* OMVs cannot be ruled out by this approach. Experimental shortcomings are possible since the experimental use of BMVs is still being established. Nonetheless, BMVs provide a novel approach to restore intestinal homeostasis and are discussed as a therapeutic strategy for IBD. Finally, a conceptual model is proposed to illustrate the potential role of BMVs in IBD pathophysiology, emphasizing the disruption in the bidirectional communication between host and microbiome.

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5 List of Abbreviations

AM	*Akkermansia muciniphila*
BMV	Bacterial membrane vesicle
BSA	Bovine serum albumin
CD	Crohn's disease
DC	Dendritic cell
DSS	Dextran sulfate sodium
EcN	*Escherichia coli Nissle 1917*
FBS	Fetal Bovine Serum
FMT	Fecal microbiota transplantation
FN/Fuso	*Fusobacterium nucleatum*
FSC	Forward scatter
GC	Goblet cell
GFM	Germ-free mice
IBD	Inflammatory bowel disease
LPS	Lipopolysaccharide
MFI	Mean/median fluorescence intensity
OMV	Outer membrane vesicle
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PG	Peptidoglycan
PRR	Pattern recognition receptor
PSA	Polysaccharide A
SCFA	Short-chain fatty acid
sGC	Sentinel goblet cell
SPF	Specific-pathogen-free
SSC	Side scatter
TJ	Tight junction
TLR	Toll-like receptor
Treg	Regulatory T cell
UC	Ulcerative Colitis

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7 Introduction

7.1 The Gut and its Microbiome are interconnected

Microbiota are found on different surface sites and body fluids of the human body, without disrupting host homeostasis. The human microbiome includes the entirety of associated microbiota, including bacteria, archaea, fungi, and viruses. Microbes are most studied for their potential to cause diseases. However, since the microbiome is also present in physiological states, increasing interest has risen to evaluate the microbiome as an essential part of a healthy host.

The interactions between host and microbiota that enable controlled reciprocity are only poorly understood. Thorough colonization of the gut occurs through postnatal contact with microbiota, which find their niche and establish a coexistence with the host. This symbiosis likely developed with mutualistic features throughout evolution (1). Host and microbiota find their mutual match and develop an inter-domain living community to benefit from each other.

The gut is specialized in interkingdom interactions since it is constantly confronted with microbiota. Up to 60% of fecal solids are bacteria (2). Microbial colonization is required for the proper development and function of the host's gut mucosa, enteric nervous system and immune system (3-6). Germ-free mice (GFM) do not develop a physiological gut mucosa capable of adequately handling pathogens or intestinal antigens. GFM exhibit underdeveloped gut-associated lymphoid tissue (GALT), accompanied by defects in regulatory T-Cells (Tregs) as well as the production of antibodies and IL-10 (4, 7, 8). The mucosa of GFM displays reduced cell regeneration of enterocytes, a longer cell cycle time (9, 10) and defective brush border differentiation (11). Additionally, villus thickness is decreased due to disturbed vascular remodeling (12).

The gut is naturally adapted to live with the microbiome, both have evolutionary developed in coexistence. In a broader context, the microbiome could be considered as a non-self organ of the human body. Molecular diagnostic tools like 16S PCR have revealed an immense diversity of the gut microbiome. Bacteria most commonly found belong to the phyla Firmicutes and Bacteroidetes. Together with Proteobacteria and Actinobacteria, these groups account for 99% of gut

bacteria (13). Anaerobic bacteria are much more common than aerobes (by a factor of 100-1000 (14)).
The composition of the gut microbiome is highly variable and depends on genetics, age and other factors. Additionally, the composition in an individual fluctuates upon external influences such as antibiotics, gastrointestinal diseases or diet (15).

7.1.1 Benefits for the Host

Bacteria express a wide variety of enzymes that complement the host's ability to digest nutrients. Carbohydrates and proteins the host is not able to digest in the upper gastrointestinal tract are met by bacteria with appropriate enzymes in the colon. Fiber not only feeds bacteria but bacterial fermentation also yields short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate as byproducts (16, 17). The host can metabolize these SCFAs. Epithelial cells require significant amounts of energy for their secretory, regenerative, and absorptive processes. Interestingly, the epithelium of the distal colon derives 60 to 70% of its energy from SCFA of bacterial source (18). SCFAs serve not only as an energy source but also as signaling molecules by binding G-protein coupled receptors, such as FFA2, FFA3, and GPR109A. One downstream effect of this is the contraction of colonic circular muscles. This is how SCFAs contribute to colonic transit in rats, which is important to prevent bacterial overgrowth (19). Additionally, the microbiome produces various vitamins including folate, niacin, riboflavin (20) and vitamin K (21).
Clinical experience and the according data have shown that antibiotic treatment can predispose individuals to gastrointestinal infections. Clostridium difficile-colitis for example thrives when the physiological microbial environment is disrupted. The commensal flora is connected by well-established and optimized enzymatic chains, leaving little opportunity for pathogens to compete for nutrition. Bacteria also produce antimicrobial substances that target other unwelcome bacteria (22). The microbiome protects its host from infections via colonization resistance.
The gut microbiome also influences many host functions beyond the gut. For example, bile acid transformation and reabsorption depend on bacterial activity and glucose sensitivity as well as bone health are also affected by the microbiome.

The gut-brain axis even links the microbiome to behavior and psychiatric conditions (23). These examples highlight the essential role of the microbiome in overall human health.

On the other hand, evolution has armed vertebrates with an innate immune system. Pattern recognition receptors (PRR) and the complement system identify molecules of bacterial origin to protect the organism from pathogenic microbes. Since this system also recognizes the physiological gut microbiome, the host requires differential response systems to maintain symbiosis with the beneficial microbiome, while also initiating defense mechanisms against pathogens. Intestinal inflammation is a result of these mechanisms failing. Cells involved in these processes are located in the gut mucosa: Epithelial cells guard the mucosa by producing a mucus barrier and controlling bacterial influx. Immune cells process microbial products and interact with the microbiome to support beneficial microbes and eliminate others.

7.2 Membrane Vesicles are ubiquitous in Nature

Membrane vesicles (MV) are bilipid-layered proteoliposomes derived from cellular membranes. These vesicles can range in size from 20 nm to 3 µm (24). A Sphere of phospholipids encapsulates a wide variety of molecules including nucleic acids, proteins and lipids. MVs are present in virtually every human body fluid, including cerebrospinal fluid and urine (25, 26). They are produced not only by eukaryotic cells, including parasites (27) and fungi (28) but also by archaea (29) and bacteria (30). Despite the diversity in cellular structures across these different branches of life, all organisms seem to have evolved their own unique mechanisms for producing vesicles from their membranes. Logically, their biological properties and contents depend on the cell of origin and the accompanying mechanism of production. This results in a big variety of MVs across different life forms.
Here we will focus on bacterial membrane vesicles (BMV), as they bridge the mucus barrier and serve as interesting communication tools for the gut microbiome to interact with its host. Metagenomic studies of stool-derived BMVs indicate that their diversity is generally lower than that of the microbial presence, suggesting that not all bacteria produce BMVs or are in a state that favors their

production. The representation of phyla is different in the BMV composition than in microbial composition. For example, Bacteroidetes are more abundant in the BMV composition, suggesting more BMV production than Actinobacteria and Firmicutes which are more present in abundance (31).

7.2.1 Biogenesis in different Bacteria

Gram-negative bacteria form membrane vesicles, known as outer membrane vesicles (OMV), by bulging and pinching off parts of their outer membrane. Most studies investigating BMVs used OMVs. Here we will use the term “OMV” for MVs of gram-negative origin and “BMV” for the entirety of bacterial membrane vesicles. Gram-negative bacteria utilize different mechanisms to separate the outer membrane from the cytoplasmic membrane (32-34). The periplasmic space, which contains peptidoglycan (PG), connects these two membranes. By modulating the activity of endopeptidases, cells can degrade PG, to release parts of the outer membrane as OMVs (35). Another mechanism of vesiculation involves the accumulation of lipopolysaccharides (LPS), proteins, or PG in the periplasmic space, creating pressure that facilitates the release of the outer membrane (36). Further, ABC-transporter systems regulate the amount of phospholipids in the outer membrane. Reduced expression of ABC-transporters can lead to an excess of phospholipids, causing bulging of the outer membrane (37).
Gram-positive bacteria were initially believed to only release membrane vesicles upon cell death since their thick murein wall surrounds the cell membrane. However, BMVs have been harvested from gram-positive bacterial cultures throughout all growth phases and without inducing cell damage (38). This observation suggests that gram-positive bacteria have their own strategies to constitutively produce BMVs (28, 39). BMVs from many gram-positive bacteria have been shown to contain autolysins, which may facilitate the passage through the murein wall (40-42), but the mechanisms of vesiculation are generally unknown.
However, gram-positive bacteria expend energy to produce MVs to even overcome barriers such as the murein wall. Vesiculation processes seem to be

well-conserved across different life forms, highlighting that these organisms benefit from MV formation.

7.2.2 Environmental Conditions determine Vesicle Biology

BMVs from the same species can vary in their composition and contents due to multiple external determinants. This variability is crucial to consider in experimental studies involving BMVs. Changes in the bacterial environment such as growth phase, media composition, and oxygen or iron availability lead to specific characteristics of BMVs (43). For example, different growth medium not only influences the amount of produced vesicles but also their protein composition (44). BMVs from different growth phases show different phospholipid-protein-ratios. In the stationary phase, the protein fraction is higher than in the exponential phase. Concordantly, the (buoyant-)density of vesicles in the stationary phase is also higher.

Generally, vesicle production is increased under stress conditions. In the host-microbe context stressors like nutrient deprivation, the host's antimicrobial peptides and oxidative stress will impact bacteria and their vesicle biogenesis (45). In these cases, vesicles can contribute to bacterial survival (46). For example, bacteria confronted with oxidative stress preferentially load their vesicles with oxidizable residues, while the cell retains transporters responsible for cellular homeostasis (47). Since different bacteria have different physiologies, external influences will not impact all bacterial species in the same way (48). The bacteriolytic properties of lysozyme, for example, depend on the structure of the cell wall and the lipid-A phenotype. Lysozyme has been shown to induce vesicle production in Pseudomonas aeruginosa. These vesicles exhibit stronger cytotoxic properties compared to those produced by bacteria grown on regular TSA plates (49).

To add another level of complexity, different stressors trigger specific forms of vesiculation and release mechanisms, which themselves contribute to the huge diversity in vesicle populations. Exposure to different antibiotics can induce distinct subsets of vesicles, which can be differentiated by their size, structure, and content (50). This is why it is important to pay attention to the origin of the vesicles.

The conditions of their formation influence their composition, which in turn determines their potential effects on other organisms.

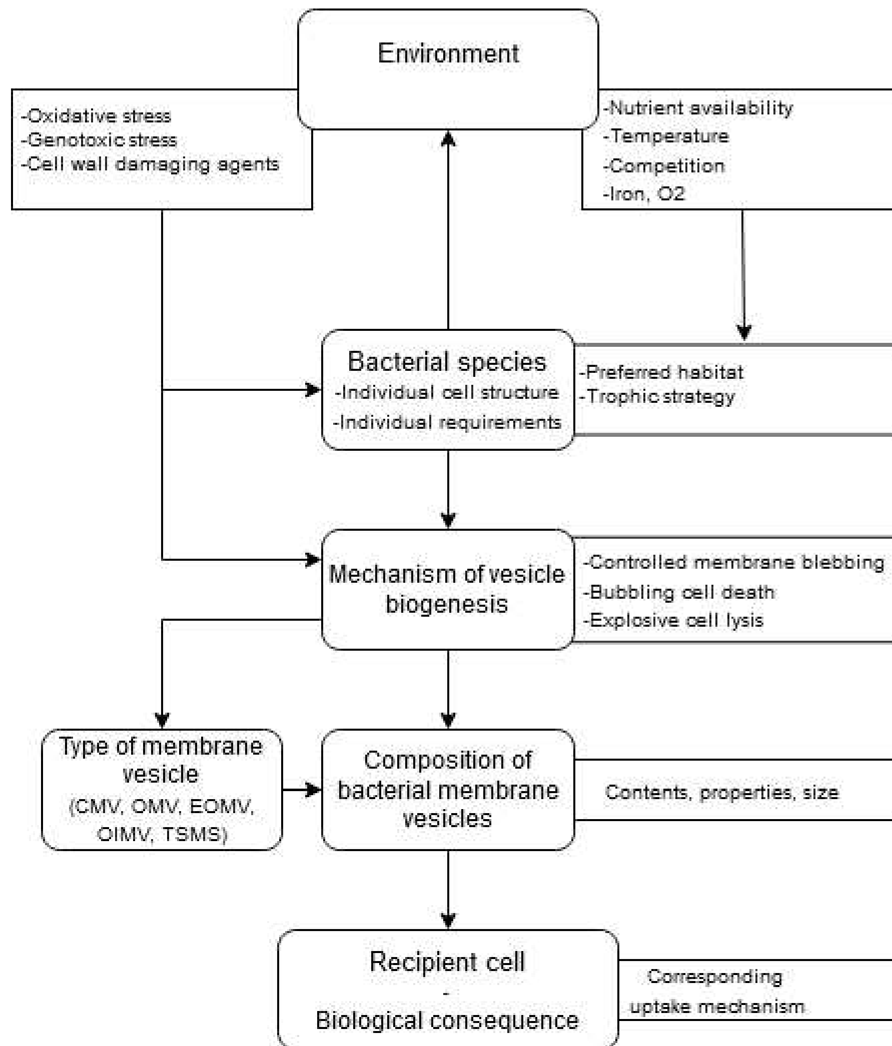


Figure 1: *The Biology of Bacterial Membrane Vesicles is complex*

Contents, size and composition of BMVs depend on numerous environmental influences. The conditions under which bacteria have produced their membrane vesicles determine their biological effects on other cells.

7.2.3 Lessons from Cell Lysis associated BMVs

When cell stress becomes excessive, bacteria undergo cell death. The cell disintegrates and forms vesicles from its remnants. Gram-positive bacteria, for

example, were observed to release MVs upon cell death when their murein wall disintegrates (42, 51). This type of vesicle formation was described in *Bacillus subtilis*, which induced the expression of endolysins under the influence of DNA-damaging substances. These endolysins degrade peptidoglycan, subsequently releasing remnants of the cell as BMVs, a process known as “bubbling cell death” (52).
Gram-negative bacteria also form vesicles upon cell death, but a different phenomenon is observed. For instance, under exogenous stress, *Pseudomonas aeruginosa* cells have been observed to round up and explode, releasing fragments that then form BMVs in a process known as “explosive cell lysis” (53).

Cell lysis-associated vesicles differ from those constitutively released under physiological conditions in both structure and content. Since the entire cell fragments into vesicles, these membrane vesicles are larger and contain more cytoplasmic materials such as DNA, endolysins and even phages (50). These membrane vesicles have different purposes. Bacteria may have evolved mechanisms to induce cell death in response to severe external stress. The membrane vesicles could serve to protect the bacterial population from further damage. For example, BMVs are able to intercept antibiotics and phages (33, 46, 54). This phenomenon can be compared to apoptosis in human cells. This process also initiates cell death to protect the greater organism and results in membrane vesicles, called apoptotic bodies.

7.3 Bacteria use Membrane Vesicles as Communication Tool

Unlike vesicles generated during cell lysis, which primarily serve as a stress response or controlled cell death, bacteria also constitutively produce BMVs supporting their ongoing survival and functionality. Initially, their role in detoxification was observed. Harmful substances are concentrated in vesicles and expelled from the cell (55, 56).
It was soon recognized that membrane vesicles do not only affect the producing cells, they serve as active communication tools, influencing their environment by interacting with other cells. The lipid membrane of BMVs protects their cargo from proteases and nucleases and prevents the dilution of bioactive molecules. When

BMVs arrive at their target cell, they can interact by binding to receptors or directly fuse with the cell membrane to deliver their contents. This form of communication has likely been preserved through evolution because it can provide advantages to bacteria in different ways.

The recipient cells can be other bacteria, facilitating the horizontal transfer of resistance or virulence genes, which helps bacteria adapt to their environment (57, 58).

Particularly relevant for the gut ecosystem is that these recipients are not limited to the bacterial species or even to the same kingdom of life, making BMVs a versatile interkingdom communication tool (59). Co-cultures of Caco-2 cells and peripheral blood mononuclear cells (PBMCs) effectively simulate the structure and function of intact intestinal mucosa. In this model, Caco-2 cells form an epithelial monolayer guarding the underlying PBMCs. When bacterial lysates from the probiotic *Escherichia coli Nissle 1917* (EcN) are administered on the apical side of the epithelium, PBMCs do not react with an immune response, demonstrating that the functional epithelial barrier prevents the passage of soluble bacterial components. In contrast, membrane vesicles from EcN stimulate an immune response in PBMCs. This highlights their role as a potent communication tool capable of controlled interaction with the host (60). Today, we even replicate this natural communication system in the form of nanoparticles loaded with bioactive molecules of interest, creating vaccines or cancer immune therapies.

For different types or sizes of vesicles, different uptake mechanisms in mammalian cells have been observed. In epithelial cells, for example, various endocytosis mechanisms internalize BMVs. These include clathrin-dependent endocytosis, caveolin-mediated endocytosis, macropinocytosis, and non-caveolin, non-clathrin-dependent endocytosis. Membrane fusion and lipid rafts have also been identified as entry routes. Phagocytic cells use phagocytosis instead. It is likely that the biological properties of the vesicles influence their uptake and interaction mechanisms with cells (61-64).

The uptake mechanism in turn determines the further downstream processing of BMVs. For example, the caveolin-dependent pathway bypasses fusing with lysosomes, which possibly avoids the degradation of internalized vesicles. Bacteria utilize different strategies to use their ability to directly influence human

cells with BMVs. Pathogenic bacteria use BMVs to spread virulence factors across barriers like the gut mucosa. For example, Vibrio cholerae can transfer cholera toxin to human mucosal cells without having to pass the mucosal barrier. This causes Cholera without requiring cellular invasion (65). Similarly, EHEC can release Shiga toxins via outer membrane vesicles, leading to severe gastroenteritis and, in some cases, extraintestinal complications such as hemolytic uremic syndrome (66, 67). Moreover, these vesicles can undermine the gut barrier and alter immune responses, helping bacteria evade the host's immune system. BMVs loaded with virulence factors can even cause sepsis without the presence of live bacteria (68).

7.4 The Commensal Strategy

A contrary approach for bacteria is achieving symbiosis with a host that in turn provides a hospitable environment and essential nutrients. Commensal bacteria have evolved strategies to colonize the host's gut and sustain homeostasis. The intestinal microbiota is generally kept at a safe distance from direct immune system contact. The mucus barrier prevents bacterial penetration under normal conditions. However, bacteria can establish remote communication through their BMVs by overcoming the tiny pores of the mucus layer and delivering bioactive molecules. This type of communication likely plays a key role in promoting a symbiotic relationship between the host and its microbiome in different ways.
The effects of BMVs are often pictured from an anthropocentric standpoint. In the following section, we will not only describe how the host benefits from BMVs but also which purpose they fulfill for the bacteria. Why would bacteria be interested in strengthening the barrier function of the host?
Generally, inflammation is the result when the coexistence of host and microbes is disrupted. Bacteria aim to avoid inflammation since it highly disturbs the mucosal habitat with an according depletion in beneficial commensals like AM (69). Commensal bacteria use BMVs to signal their good intent to the host.

7.4.1 Bacterial Membrane Vesicles induce Tolerance

7.4.1.1 Bacterial Membrane Vesicles carry Immunogenic Cargo

BMVs of commensal origin are observed to modulate the immune response by inducing tolerogenic and anti-inflammatory effects, thereby promoting a balanced interaction with the host's immune system. BMVs generally are bacterial products containing Pathogen-associated molecular patterns (PAMP). Pattern recognition receptors (PRR) are responsible for the identification of PAMPs as non-self to possibly initiate proinflammatory cascades.

OMVs contain LPS from their originating bacteria's outer membrane (70). The LPS characteristics of the outer membrane and the OMVs are consistent (71). LPS interacts with PRRs, such as Toll-like receptor 4 (TLR-4), to initiate both innate and adaptive immune responses. However, the immune response induced by LPS can vary based on the structure of Lipid A, including its acetylation pattern and phosphate groups. LPS may bind to PRRs without inducing an immune response, potentially antagonizing other LPS in copresence (72). Commensal bacteria use Lipid-A 4'-phosphatase to dephosphorylate LPS, resulting in a form of LPS that has reduced agonistic effects on PRRs (73). The dephosphorylation also accomplishes resistance against antimicrobial peptides, a common tool used by the host mucosa (74). In a gnotobiotic mouse model, mutants lacking lpxF were outcompeted, highlighting that LPS with reduced immunogenicity supports commensal survival. On top of that, immune cells can develop a state of reduced responsiveness, known as "microbial tolerance", when exposed to prolonged LPS stimulation. This results in decreased production of proinflammatory cytokines (44, 48). However, the response of the anti-inflammatory cytokine IL-10 to LPS remains unaffected by this tolerance. This phenomenon is also observed with other microbial molecules (75).

7.4.1.2 Pattern Recognition Receptors sense Commensals

Lately, it has become evident that the downstream effects of PRR binding are more intricate than simply defending against non-self entities. Host-microbe interactions evolutionary generated diverse bidirectional interaction mechanisms. Sensing molecules of bacterial origin is also essential to recognize beneficial bacteria and treat them as such. Even in homeostasis and without clinical

inflammation the gastrointestinal mucosa is full of immune cells, “physiological inflammation”, representing constant immune responses to microbiota-derived antigens due to persistent exposure. NOD-receptors as soluble intracellular PRRs sense bacterial peptidoglycan. NOD signaling is associated with different outcomes like autophagy, production of antimicrobial peptides and IgA or stabilization of Tregs (76). All of this is crucial in gut mucosal homeostasis. Interestingly, NOD1 is most prominently expressed in the gut epithelium (77). Commensal BMVs were shown to be sensed by different Toll-like-receptors as well as NOD1 and NOD2. OMVs of probiotic *Escherichia coli Nissle 1917* (EcN) and commensal ECOR12 for instance, induce cytokine responses in a NOD1-dependant manner. They deliver their peptidoglycan to intestinal epithelial cell lines via endocytosis (78).
Many of the following studies have highlighted the importance of PRRs in implementing the effect of BMVs on the host.

7.4.1.3 Immune Cells process Bacterial Membrane Vesicles

Dendritic cells (DC) are specialized in host-microbiota interplay. Their pseudopodia can reach though the intestinal epithelium actively getting in contact with the microbiome environment. They express PRRs to process bacterial molecules, including BMVs, and initiate an adequate response by their differential influence on other immune cells.
OMVs of commensal *Bacteroides vulgatus* induced semi-mature tolerant DCs from bone marrow-derived DCs (79). Unlike untreated DCs, these smDCs did not respond with elevated expression of TNF-α and MHC-II following stimulation with pathogenic *E. coli*. Both TLR-2 and TLR-4 were simultaneously needed to exert this effect. Bacteria use BMVs to prime DCs, modulating their differential response to bacteria.
DCs relay microbial information to engage other immune cells by presenting antigens. Dendritic cells stimulated with ECOR12- or EcN-OMVs activate native T-cells while inducing balanced immune responses (80). BMVs promoted changes in surface antigens, secretory responses and costimulatory molecules expressed by DCs to facilitate T-Cell activation.
Regulatory T-Cells (Treg) mediate intestinal homeostasis by maintaining tolerance to harmless environmental influx (81). FoxP3 is the driving transcription factor for

Treg development. Humans with mutations in FoxP3 suffer from severe intestinal inflammation (82). Commensals are known to be able to stimulate Treg development. Accordingly, germ-free mice (GFM) exhibit reduced Treg populations (83). One key effector mechanism utilized by Tregs is the production of the tolerogenic cytokine IL-10 which suppresses other immune cells and dampens their cytokine production (84). Tregs are the main producers of IL-10 in the colon (85). When IL-10 production by Tregs or the IL-10 receptor is dysfunctional, mice develop spontaneous colitis (86-88). Interestingly, GFM deficient in IL-10 do not develop colitis (89), underscoring that IL-10 is important for maintaining homeostasis in naturally microbe-exposed sites such as the intestine (84). IL-10 is an essential mediator of intestinal homeostasis.
Bacteroides fragilis OMVs induce tolerogenic IL-10-producing DCs. This effect largely depends on the molecule polysaccharide A (PSA), which is carried by OMVs (90). DCs use TLR-2 to sense PSA and initiate downstream Gadd45α signaling to induce an IL-10 response. OMV-pulsed DCs subsequently stimulate the generation of Tregs from native T-cells through the secretion of IL-10. Tregs increase FOXP3 expression and IL-10 secretion and show enhanced suppressive capacity in cell culture suppression assays. In addition to TLR-2, DCs also require ATG16L1 and NOD2 to promote Tregs in response to *B. fragilis* BMVs (91).
In vivo studies have shown that *B. fragilis* OMVs alleviate experimental colitis in mice (92). This in vivo effect was associated with an increased production of IL-10 among regulatory T cells and a reduction of IL-17A.
Further studies with *B. fragilis* gave interesting insights into the molecular processing of OMVs and their PSA. T-cells respond to purified PSA with IL-10 production and FOXP3 expression but not to OMVs containing PSA. The OMVs are processed by DCs and PSA is displayed via MHC-II to T-cells which in turn require TLR-2 to sense PSA and react appropriately (8, 93).
ATG16L1 is best known for its role in regulating antibacterial autophagy (94), and polymorphisms in ATG16L1 can lead to impaired microbial clearance. But ATG16L1 polymorphisms are also associated with abnormalities in goblet and Paneth cells and cytokine responses. Mice deficient in ATG16L1 develop spontaneous chronic intestinal inflammation characterized by a reduction in Foxp3+ Tregs and an expansion of Th2 cells (95). ATG16L1 was shown to be

involved in OMV-mediated, DC-dependent induction of Tregs, highlighting the complexity of components involved in microbial interaction (91).
The host can profit from *B. fragilis* OMVs, but bacteria and evolution are not altruistic. Studies on PSA illustrate how *B. fragilis* can profit from modulating the immune response of their host.
Colonization with *B. fragilis* defective in PSA expression leads to reduced numbers of mucosa-associated bacteria. Treatment with PSA was able to reverse this. *B. fragilis* utilizes PSA-loaded OMVs to establish colonization in its mucosal niche in mice (93). This chain of events is most understood for *B. fragilis* OMVs, but other commensals seem to use similar approaches.
The host has to adequately process these bacterial signals to support commensal colonization. Animals lacking TLR-2 in T-Cells show similar reductions in mucosal colonization since they are unable to process PSA.

Different non-pathogenic *Escherichia coli* strains isolated from healthy humans differentially activate dendritic cells and modulate T-cell responses (96). The commensal strain ECOR12 led to higher responses in IL-10 and TGF-β compared to other *E. coli* strains. *Escherichia coli Nissle 1917* (EcN) is a probiotic strain isolated from a soldier who did not develop infectious gastroenteritis like his peers. This strain is associated with an increased production of TH1-inducing cytokines, which may contribute to its protective effects against infections. These findings highlight the biological diversity of BMVs, as even different strains of the same species can elicit distinct responses. RNA deep sequencing revealed that BMVs from ECOR12 and EcN induce their immunomodulatory effects in human dendritic cells, at least partly through alterations in their miRNA profiles (97). A group of miRNAs is upregulated upon treatment. These miRNAs are known to mediate DC-maturation, tolerogenic responses and cytokine-finetuning. Macrophages are also influenced by EcN-MVs. When RAW264.7-macrophages are treated with EcN-MVs, IL-10 induction is higher than proinflammatory responses of TNF-α and IL-6 (98). Following stimulation, the macrophages were also observed to enhance their antibacterial activity when exposed to pathogenic bacteria.
These indications of anti-inflammatory effects were evaluated in DSS-colitis mice (99). Pretreatment with EcN-OMVs before inducing colitis ameliorated clinical

symptoms like body weight loss, bleeding and diarrhea. Disease severity markers like colonic weight/length ratio as markers for colonic edema and histologic inflammation were improved. On a molecular level, many different proinflammatory cytokines were dampened, while IL-10 was higher compared to untreated colitis. The immunomodulatory responses to EcN-BMVs explored in in vitro experiments may explain the improvement of colitis.

BMVs from commensal *Bacteroides thetaiotaomicron* also elicit cytokine responses in human colonic biopsies, including the regulatory cytokine IL-10 (100). After OMV treatment the ratio of lymph follicle-DCs expressing IL-10 and IL-6 shifted towards IL-10. Absolute numbers of DCs expressing IL-10 were elevated, while the amount of IL-6 expressing DCs stayed stable after treatment. Commensals induce PPAR-γ in their host (101) to promote homeostasis by antagonizing NF-κB and increasing the transcriptional activation of IL-10 (102). *Faecalibacterium prausnitzii* BMVs induce different PPARs, with the most prominent being PPAR-γ (103). A decrease in PPAR-γ is associated with an increased susceptibility to colitis (104).

Akkermansia muciniphila (AM) is another important gut symbiont and will be introduced later in this thesis. Its OMVs induce IL-10 expression but additionally reduce TNF-α expression in the intestinal mucosa of mice (105). AM-OMVs also mitigate disease severity in IBD mouse models. But this effect may not be solely attributed to their immunomodulatory properties (106).

7.4.2 Bacterial Membrane Vesicles reinforce the Mucosal Barrier

Bacteria have evolved various strategies to inhabit the host's gastrointestinal tract. The coexistence of host and microbiome is only possible because both are spatially separated. Commensals benefit from an intact host barrier and even reinforce it to conserve homeostasis.
Intestinal stem cells renew the epithelium every 3-4 days. The epithelium consists of different cell lineages: absorptive enterocytes with microvilli upscaling surface area, enteroendocrine cells, mucus secreting goblet cells and Paneth cells. Absorptive cells are most abundant in the small intestine specializing in nutrient absorption, while mucus secreting cells are more common in the colon where

bacterial load is higher and stool has less water content. In the colon cells arise from the base of the crypts and differentiate while migrating towards the lumen (107, 108).
Tight junction (TJ) proteins seal the epithelial barrier by tightly attaching the cells to avoid uncontrolled paracellular influx from the intestinal lumen. Important TJ proteins include occludin and claudin, along with associated proteins such as ZO-1, which anchor TJ proteins to the cell membrane.
Barrier function is crucial for maintaining physiological interaction with microbiota. Intestinal epithelial cells illustrate this. They express TLRs nearly exclusively on their basolateral side, allowing for apical bacterial presence and controlled interaction. When tight junctions and other barrier components fail, their basolateral side will be exposed to microbial products causing uncontrolled inflammatory responses (109).
Akkermansia muciniphila (AM) is a common anaerobic mucin-degrading commensal associated with numerous benefits for the host and is currently investigated for its therapeutic potential. LPS-induced tight junction permeability was decreased by AM-OMVs in Caco-2 cells (110).
These barrier-strengthening effects could be replicated in vivo. High-fat diet mice suffer from increased gut permeability. After oral administration of AM-MVs, barrier function in these mice was shown to improve in in vivo permeability assays. This was attributed to the upregulation of occluding and claudin-5 through AMPK phosphorylation. Additionally, less damage to the barrier was histologically observed. AMPK, previously known for its role in regulating tight junction assembly, was key in this process (111).
Another study involving obese mice on a high-fat diet found that AM-MVs increased mucosal thickness and reduced gut permeability. This effect was associated with the upregulation of ZO-1, occludin, and claudin-1, as well as the downregulation of claudin-2, a protein associated with increased barrier permeability due to its pore-forming properties (112).
Finally, DSS-colitis mice cotreated with AM-OMV showed less inflammation and improvements in epithelial stability in histologic assessments. Clinical symptoms and disease markers were alleviated (106). AM-OMVs are further processed in Payers patches, where they induce an IgA response (113). IgA is essential to coat

the intestinal microbiota, extruding pathogens while enabling colonization with commensals and thus creating an immune barrier (114, 115).
In T-84 and Caco-2 monolayers OMVs from EcN and commensal ECOR63 were shown to upregulate mRNA of TJ proteins *ZO-1* and *claudin-14* and downregulate *claudin-2*. Induced expression of these genes resulted in enhanced transepithelial resistance (116).
Infection with enteropathogenic *E. coli* provides another cellular model of epithelial barrier dysfunction with downregulated ZO-1, ZO-2, occludin, claudin-14 and significant translocation of TJ-proteins to the cytoplasm. EcN-OMVs prevented this disruption by their ability to retain ZO-1 and occludin in TJ structures. Furthermore, the mRNA levels of claudin-14 and occludin were elevated, and the disorganization of the F-actin cytoskeleton was prevented (117). EcN-OMVs alleviate disease severity in IBD mouse models (99), a benefit associated with restored levels of TFF-3, which facilitates mucosal repair and epithelial migration and suits as a marker for intestinal barrier function (118). Mice defective in TFF-3 exhibit impaired barrier integrity and mucosal repair and as a result are more susceptible to DSS colitis (119). In addition, MMP-9, which correlates with intestinal inflammation, disease severity and TJ disruption, was downregulated (120).
Faecalibacterium prausnitzii is another notable commensal bacterium residing in the human intestine. When Caco-2 cells are challenged with its OMVs, ZO1 and occludin mRNA are induced (103).

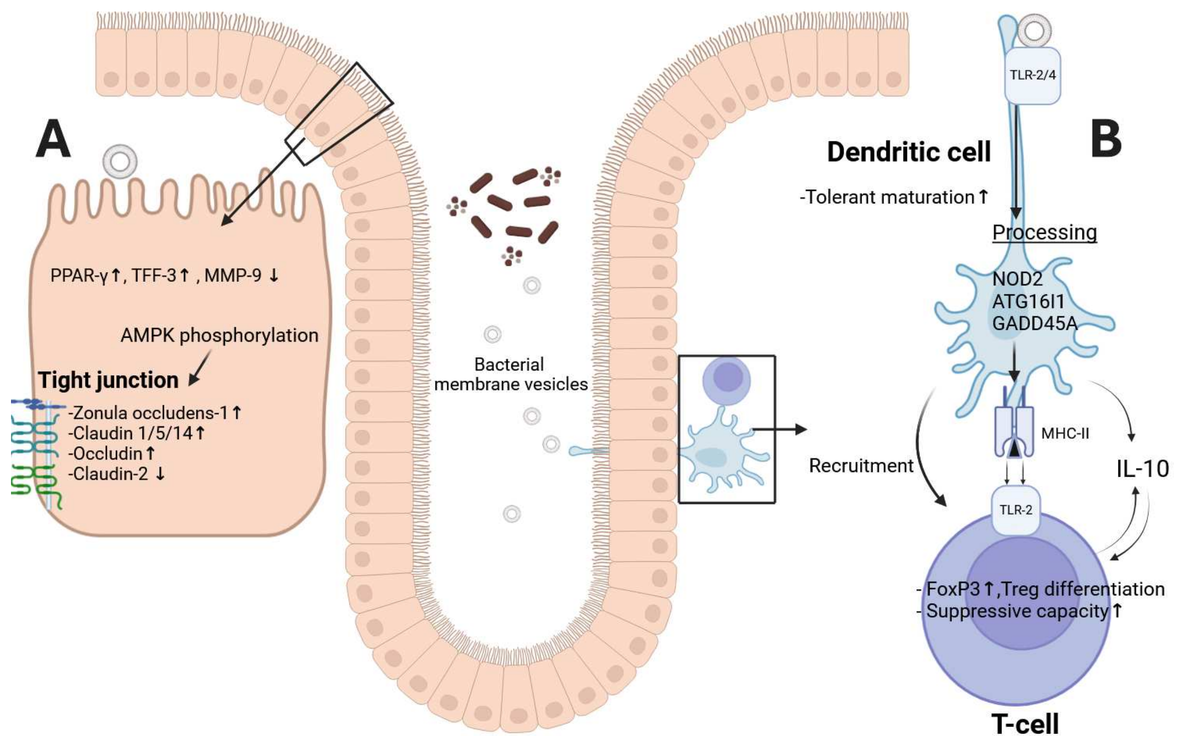


Figure 2: *Bacterial Membrane Vesicles mediate Homeostasis*

A: BMVs reinforce the epithelial barrier to achieve spatial segregation
B: BMVs are sensed and processed by the immune system to induce tolerant immune responses.

7.5 Mucus separates Host and Microbes

The host barrier extends over the epithelial outline. The epithelium is coated with mucus, an aqueous viscous gel-like secretion produced by goblet cells. In the digestive tract mucus is an essential lubricate for stool transit and achieves separation of epithelium and bacteria. Nonetheless, mucus is not a strict barrier, but rather an exchange place where nutrient uptake and host-microbe interactions happen under controlled circumstances.
Mucin proteins give mucus its typical structure. Mucins are large highly glycosylated (up to 80% carbohydrate (121) proteins (i.e. proteoglycans). Their concentration in mucus is only 1-5% because of their high potential to bind water (122). Around 97% of mucus consists of water. Other proteins, salts, lipids and debris make up for the rest of the solids. Its high water content makes it an

excellent solvent for important proteins like enzymes, antimicrobial proteins, growth factors and immunoglobulins. The mucus layer avoids dilution and loss of these important effector molecules into the intestinal lumen.

To date, 21 different mucin genes have been discovered. Different organs of the body express their specific consortium of mucin proteins. Thus airway-, digestive- and reproductive tract differ in their mucin protein profiles. There are two types of mucins.

Transmembrane mucins contain an extra transmembrane domain which anchors the glycoprotein to the apical cell membrane instead of being secreted (123). MUC7 for example was shown to multimerize which avoids water-binding properties (124) indicating other functions than in secreted mucins. Epithelial lineages express transmembrane mucins to coat their cell membrane. The N-terminal extracellular domain contributes to the dense glycosylation and mucus coating of the epithelium.

The function of these non-secreted mucins is less understood, but they appear to play a role in protecting against bacterial adhesion (125). For instance, MUC1-deficient mice have shown increased susceptibility to Helicobacter pylori colonization and subsequent gastritis (126). Their role in interactions with commensals is also discussed.

Transmembrane mucins include MUC 1, 3A, 3B, 4, 11, 12, 13, 15, 16, 17, 20 and 21.

On the other hand, gel-forming mucins are secreted and play a crucial role in forming the mucus layer. Different mucins are expressed along various segments of the gastrointestinal tract. For example, the gastric mucosa primarily expresses MUC5AC and MUC6. In the duodenum MUC2 and MUC6 are expressed in Brunner`s glands. While the small intestine and colon also have traces of other mucin proteins such as MUC5B and MUC2, the latter remains the predominant mucus-forming protein (124).

Goblet cells fulfill the mucus secretory function of the intestinal epithelium. Goblet cells are characterized by their ability to produce, store and secrete MUC2. Other epithelial cells of the intestine do not express MUC2. After biosynthesis, MUC2 is

stored in secretory granules that occupy most of the cytoplasm, giving the goblet cell its characteristic morphology. In MUC2-deficient mice, goblet cells cannot be identified (127).

7.5.1 Biosynthesis of MUC2

After translation, the mucin protein undergoes N-glycosylation and dimerization via disulfide bonds between its C-termini in the endoplasmic reticulum. Hereafter O-glycosylation in the Golgi-apparatus starts with adding N-acetylgalactosamine. During migration through the Golgi, the dimers meet multiple specific glycosyltransferases which continue glycan extension. Different cells and species express different glycosyltransferases, which leads to a big variety in glycosylation profiles of mucin proteins (121). Glycosylation forms a glycan coat around the protein core in a bottle brush configuration which protects the core from microbial proteases and digestive enzymes. This configuration provides an immense surface site of glycans which bind water. Enzymes adding fucose or N-acetylneuraminic acid stop further glycosylation and terminate elongation.
The *O*-glycosylation of colonic mucins is highly variable with different core glycans, variable side-chain sizes, branches, and multiple capping structures such as sulfate, sialic acid, fucose, sulfate and acetyl groups. Capping structures protect the mucin O-glycans from degradation. O-glycosylation is essential for the structural and functional integrity of mucin proteins. Mice defective in O-glycosylation or sulfation lack the protective function of mucins and develop spontaneous chronic colitis (128, 129).
After oligomerization through their C-termini, the trans-Golgi apparatus organizes MUC2 glycoproteins into secretory vesicles (130, 131). In secretory vesicles, MUC2 is kept at low pH and high calcium concentration. In this milieu, the MUC2 molecules are densely stacked (132, 133).

7.5.2 Mucin Secretion

The secretory vesicles merge with the cell membrane and extrude the mucins. This process is called regulated vesicle secretion and facilitates continuous mucin secretion. Recently, a different form of mucin secretion has been observed, compound exocytosis (134). In this case, the apical cell membrane ruptures to rapidly release the whole reserve of mucin vesicles. This observation lead to the

discovery of different subpopulations of goblet cells (GC).
In contrast to intercrypt GCs which specialize in continuous mucin secretion and the formation of the inner mucus layer, sentinel goblet cells protect the crypt opening by providing situational mucin-secreting mechanisms (135). They respond to TLR-ligands with an activation of the NLRP6 inflammasome. The associated Ca^{2+}-signal results in compound exocytosis. This cellular Ca^{2+}-signal is spread to adjacent GCs via gap junctions, causing simultaneous excretion of secretory mucin vesicles. It is a protective mechanism with short-term benefits since activated sentinel GCs are expelled from the epithelium.
Exocytosis releases the mucins to interact with the apical extracellular milieu. Here the pH is significantly higher due to action of bicarbonate transporters and, the calcium concentration is also decreased. Both distinctions are required to transform the storage configuration of MUC2 into an organized net. Mice with defects in the CFTR-ion channel do not properly secrete bicarbonate which leads to attached and dysfunctional mucus (136). Under physiological conditions, the mucin structure expands to yield a highly increased surface, which significantly enhances its water-binding capacity (133, 137). This network of mucin proteins forms a sieve capable of excluding bacteria that are larger than the pores.

7.5.3 Structure of the Mucus Barrier

MUC2 forms the mucus layer in both the small intestine and the colon, but its properties vary between these regions. In the small intestine, a single layer of mucus fills the spaces between the villi and covers their tips. Since nutrient digestion and absorption require direct contact with luminal contents, the mucus in this region is loose, leaky and easily aspirated. While these properties facilitate nutrient absorption, they also increase susceptibility to bacterial invasion. Indeed, beads of the size of bacteria can penetrate this less dense mucus layer (138).
The small intestine uses a different barrier strategy. Paneth cells are unique to the small intestine and located at the base of crypts. They secrete antimicrobial proteins to prevent bacteria from getting in direct contact with the host.
As the bacterial load increases from the stomach to the rectum, the need for effective separation between the host and microbiota becomes more critical. Consequently, the mucus layer typically thickens along the gastrointestinal

tract (139, 140).
Besides its increased thickness, the mucus in the colon exhibits a distinct structural organization. It comprises two layers: the inner mucus layer is tightly adhered to the epithelial surface and forms a dense, impenetrable barrier to bacteria. This inner layer creates a network of pores that are smaller than bacterial cells (down to 0,5µm (132)), creating a physical barrier. Thus, the inner mucus layer remains free from bacteria, protecting the underlying mucosa (141). The outer layer seems to develop and expand from the inner layer due to proteolytic enzymes. Microbial proteases might support this process but GFM also show two distinct colonic mucus layers. The outer mucus layer is more penetrable, less dense and serves as a microbial habitat.
Despite their different properties, proteomic studies have shown that both the inner and outer layers are similar in their protein composition (141). But recent studies have shown that these layers differ in their glycosylation profile and even stem from different origins. The proximal and distal colon produce distinct glycosylation patterns and their respective mucin has its own purposes. Mucus-encapsulating fecal pellets along the colon is entirely of proximal origin. This encapsulation creates another microbial habitat covered by mucus (142).

7.5.4 Mucus enriches Microbial Life

The O-glycans of MUC2 serve as attachment sites for bacterial adhesion molecules and as a nutrient source, making mucus an attractive habitat for bacteria equipped with the appropriate tools. *A. muciniphila* (AM) is a common member of the outer mucus layer. When AM colonizes GF mice, it will most abundantly settle in the cecum, where mucus production is highest (143).
Many bacterial species express carbohydrate-active enzymes like glycoside hydrolases, sulfatases and esterases, which degrade the glycosylated MUC2. Just as mucin biosynthesis requires various enzymes, the cleavage of the specific linkages does as well. AM is specialized in mucin degradation and expresses all the necessary enzymes, enabling it to grow on mucus alone. Other bacteria lacking this repertoire can complement each other to create metabolic chains, finally utilizing the mucin O-glycans as a carbon source (144). However, the repertoire of enzymes must match the glycosylation profile of mucin.

Commensalism is again achieved by finding a suitable match. For instance, AM can grow on porcine gastric mucins but fails to replicate on pig colonic mucins with different sulfation patterns (145). Mice with mutations in their *MUC2* gene exhibit intestinal dysbiosis (146) and a loss of host O-glycans changes microbial composition and its metabolic output. Such disruptions in the microbial niche circle subsequently affect the host since the host transcriptome is changed leading to a disturbed homeostasis and cell regeneration (142). Mucin-degrading commensals and their associates prefer to settle in mucus-enriched environments. Mice with defective O-glycosylation exhibited an altered microbial composition with a reduced abundance of mucin-degrading commensals (142).
Mucin-degrading bacteria are essential to feed other microbial peers when dietary fiber is lacking. Bacteria that do not express the appropriate enzymes fail to grow when cultured on mucus alone. This includes *Anaerostipes caccae*, *Eubacterium hallii* and *Faecalibacterium prausnitzii*. When the same bacteria are joined by AM, they thrive and become metabolically active. AM provides glycan fragments for its partners and, in return, benefits from the supply of vitamin B12. This arrangement highlights the concept of syntrophic mucin degradation. Mucus and its metabolization are essential to sustain the microbial community in times of limited nutrients (147, 148).
The composition of the gut microbiome is still strongly influenced by diet, which determines nutrient availability. Transcriptomic studies reveal that microbes react to such changes by adapting their expression profile to the nutrient availability (149). But this is not always sufficient: A fiber-free diet leads to a decline in fiber-degrading bacteria while increasing the abundance of mucin-degrading bacteria. Interestingly, a fiber-free diet also correlates with a thinner mucus layer (150). It was speculated that bacterial mucus degradation may in this case surpass its production. Pathogenic species like *Entamoeba histolytica,* a protozoan parasite, indeed uses mucin-degrading enzymes to invade the intestinal mucosa (151). But paradoxically, commensal mucin-degrading bacteria, including *A. muciniphila*, are associated with an increased mucin abundance. Commensals use the same utility to establish their mucosal niche. For example, deleting key mucin-degrading enzymes (e.g. BF3134, BF3134) in *B. fragilis* impairs its colonization of the outer mucus layer. Only when *B. fragilis* reaches its niche in close proximity to the host,

it ramps up its metabolic activity and protein secretion. Commensal bacteria use mucin-degrading enzymes to establish communication with the host, exerting their homeostasis-supporting effects. This was illustrated by an interesting study in DNBS-colitis mice, wherein the PSA-mediated anti-inflammatory effects of *B. fragilis* depend on its mucin-degrading capacity (152).
Since not all bacteria are able to acquire this mucosal niche for themselves, microbial populations differ between the mucus layer and the intestinal lumen (153-155). But some species inhabit both compartments, as they are physically connected. But depending on the compartment, their gene expression differs. The intestinal mucus layer is its own microbial niche with distinct requirements (152, 156).

7.6 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) include ulcerative colitis (UC) and Crohn's disease (CD). These chronic inflammatory conditions of the gastrointestinal tract significantly reduce the quality of life in patients of all ages. Patients experience symptoms like diarrhea, rectal bleeding, weight loss, joint pain and psychiatric symptoms. Since the prevalence of IBDs is increasing particularly in urbanized regions (157, 158), environmental factors are suspected to trigger the disease. External influences such as hygiene, lifestyle and diets low in fiber have an impact on the gut microbiome, which may be the reason for their association with the disease. But the pathogenesis of IBD is complex, multifactorial and not yet understood. Immune dysregulation, (epi)genetics, barrier dysfunction and microbiome alterations are processes involved in the disease. The most important characteristic of IBD is that these processes are interconnected. Disruptions in one of them will have an impact on the others (159, 160). This sustains inflammation, disease progression and may explain their tendency for recurring active disease phases. Another contributing factor is inflammation itself, which exacerbates barrier dysfunction, promotes shifts in the microbiome and impacts host epigenetics. These interconnections highlight the complexity of IBD pathophysiology. There are still ongoing debates about the primary causes versus secondary consequences of the disease. But as we can see, even secondary

phenomena could sustain the disease by feeding the vicious cycle surrounding inflammation.

7.6.1 Mucus and Inflammation

Firstly, an impaired mucus barrier can lead to inflammation because bacteria and mucosal cells come in direct contact. Mice deficient in *MUC2* (−/−) fail to grow and develop properly. In these mice, goblet cells are not present, the colonic architecture is severely disrupted and they spontaneously develop colitis. Additionally, these mice are more susceptible to dextran sulfate sodium (DSS), which is used as a model for colitis (161). But even when *MUC2* is expressed, missense mutations or mutations in glycosylation enzymes can impair mucus functionality. This also compromises the integrity of the mucus barrier and leads to spontaneous colitis (146, 162).
The DSS-colitis model in mice causes intestinal inflammation as DSS disrupts the mucus barrier (163). Under physiological conditions, the inner mucus layer is capable of preventing bacterial penetration. DSS decreases mucus thickness and increases its permeability, allowing bacteria to reach the epithelial cell surface (135, 164). Bacteria invading the inner mucus layer trigger inflammation via inflammasome responses. Germ-free mice challenged with DSS exhibit only mild colitis, further confirming this connection (165).
Now we have established that an insufficient mucus barrier can cause inflammation. But colonic inflammation is inherently associated with defects in the mucus layer, regardless of the underlying cause of inflammation (166) The reverse causation however, how inflammation affects the mucus barrier, is less clear.
The relationship between mucus and inflammation appears to be time-dependent. In acute inflammation, goblet cell differentiation and mucin secretion can be increased (167). In response to inflammation, the mucosa initiates counteractive mechanisms that include the induction of mucus production. The regulation of mucin production and secretion is complex and not fully understood. In gastrointestinal inflammation the transcription factor nuclear factor NF-κB is upregulated by different pathways in addition to MAPK signaling, TNF-α and several cytokines. NF-κB binds to the promotor of *MUC2* and induces its expression. Several prostaglandins contribute to this stimulation via cAMP

signaling and the transcription factor CREB (168). On the other hand, TNF-α was shown to reduce the number of goblet cells in colonoids (169).

To further protect against bacterial invasion, bacterial molecules like lipoteichoic acid, LPS and flagellin A stimulate mucin production (124, 170). When bacteria try to enter crypts, sentinel GCs induce compound exocytosis in surrounding goblet cells, spilling the stored mucus to flush the crypt. SGCs sense bacterial LPS, but only trigger when a threshold of LPS is present. This defense mechanism is effective for short-term events like self-limiting gastrointestinal infections. But when the cause of inflammation exceeds a short time frame, these protective mechanisms can become depleted. Continuous LPS stimulation leads to repeated activation and eventually depletion of sGCs, which require time to renew (171). This explains how chronic inflammation results in the depletion of goblet cells and a thinned mucus layer (167).

All of these different mechanisms are at play and complicate the in vivo situation. The complex reality is illustrated by studies examining the mucus system in IBD since they often presented divergent results. In IBD patients many of these variables come together. The severity of inflammation, disease localization, immunosuppressive treatments, or other individual factors all influence mucus characteristics. As highlighted above, active and chronic inflammation impact mucus features differently. Flares of acute inflammation and ulceration alternate with chronic inflammation and mucosal regeneration. Since inflammation itself disrupts the mucus physiology, pathological features strongly depend on the sampling site and timing. But even in remission, bacteria in IBD patients are more closely associated with the epithelium, indicating barrier defects that persist independent of inflammation (172).

The mucus system can be characterized by multiple variables, such as goblet cell counts and functionality, *MUC2* gene expression and secretion, mucus thickness or permeability and glycosylation status. This leads to further confusion since most studies focus on only a subset of these aspects. The mucus system of IBD patients may be affected differently in individuals. Isolating mucus parameters or disease conditions will never fully capture the complex in vivo situation in IBD. Complex diseases are too intricate to study as a whole, yet too complicated to simplify.

In inflammatory bowel diseases, chronic inflammation meets defects in the mucus barrier and an increased association of bacteria and mucosa (166).
The general consensus is that the mucus barrier is impaired in IBD, which likely contributes to its pathogenesis. However, both entities are affected differently.

7.6.2 Mucus in Ulcerative Colitis

In active UC the mucosal layer is thinner, more variable and in part denuded. These changes are pronounced upon UC-related inflammation (173-175), correlate with disease severity and are associated with increased bacterial migration towards the epithelium (176). Patients with severe UC show a deficiency of MUC2 expression (177). Yet most studies find no difference in *MUC2*-mRNA expression compared to healthy controls (178, 179). However, protein levels of MUC2 and its precursor are reduced, suggesting a translational defect. MUC2 staining is reduced on the epithelial surface as well as intracellularly. These defects in the mucus barrier are also displayed in colon segments without inflammation (180).
The reduction of MUC2 protein (181) is also evident in remission, strongly suggesting these effects are not merely an epiphenomenon of inflammation. Besides quantitative defects, a subset of UC patients exhibited more penetrable mucus. This group has reduced membrane expression of SLC26A3, which is responsible for bicarbonate secretion. Bicarbonate is required for the proper unfolding and interconnection of MUC2 (180).
Defective MUC2 is accompanied by aberrant GCs. UC patients show depleted GC counts and GCs in UC patients contain fewer mucin granules (175, 182). Other epithelial cells in inflamed and non-inflamed sites of the colon are filled with atypical membranous vacuoles (183).
The stem cell niche is affected by a reduction in GC precursor cells before inflammation occurs. These findings are consistent with observations in DSS-colitis (184). Gersemann et al. showed reduced GC differentiation factors (KLF4 and HATH1) in UC patients, possibly explaining the reduced GC counts (182). The number of sentinel goblet cells (sGC) is also reduced. TLR2 ligand P3CSK4, a trigger of compound exocytosis, increases mucus secretion in control and remission UC biopsies but not in active UC, revealing defects in sGC function

during disease (180). The authors hypothesized that in disease conditions sGCs are continuously challenged because of increased bacterial influx and finally depleted since they lack proper recovery and storage of MUC2. SGCs require NLRP6 to initiate mucus secretion upon bacterial stimulation. In UC the expression of NLRP6 is reduced in the epithelial layer (176). The epithelium also contains fewer and non-glycosylated MUC2 precursors with an aberrant cytoplasmatic localization. Morphologically, the GCs are immature (177). Moreover, the glycosylation pattern of MUC2 in UC patients is distinctly different from controls (183). Glycosylation is essential for the barrier capabilities of mucins, and sulfation of colonic mucin protects the glycoprotein from uncontrolled degradation. In UC, MUC2 is hypoglycosylated, less complex, and lacks appropriate sulfate capping (185, 186).

Colonoids ramp up the expression of glycosylation enzymes upon differentiation but in colonoids from UC patients, some glycosylation enzymes fail to develop. Such defects at the post-transcriptional level may account for altered glycosylation patterns (169).

In contrast, the glycosylation pattern in UC patients in remission resembles that of healthy controls. In one study, a patient with an aberrant glycosylation profile was followed up and finally showed normalization of the glycan profile upon reaching remission (178). Defective glycosylation appears to be linked to inflammation and is reversible.

These post-transcriptional defects particularly impact mucins because of their large and complex structure (187). Proteins are misfolded and aggregate in the endoplasmic reticulum and Golgi apparatus. The accumulation of unfolded mucin precursors results in ER stress triggering unfolded protein responses and subsequently inflammation and apoptosis. ER stress itself leads to defective post-transcriptional modification of mucins and shortened GC lifespan. Inflammation and mucin defects create a self-sustaining cycle that impairs barrier function (183).

Mucus characteristics in remission appear very heterogeneous and highlight that IBDs are multifactorial and very individual. The mucus barrier of some UC patients is comparable to healthy controls, while others exhibit penetrable mucus similar to the active UC group (163).

Investigating the behavior of the mucosa of UC patients in the absence of inflammation can provide more independent insights. For example, intestinal stem cells from UC patients can grow into colonoids. Even colonoids originating from unaffected tissue were unable to produce a physiological mucus layer, showing that inflammation is not the only disturbance. GCs of healthy subjects secrete MUC2 in response to cholinergic or PGE2 stimulation, but UC-colonoids fail to respond accordingly (169).
Even in remission, the mucosa of UC patients differs in its gene expression. The intestinal epithelium exhibits multiple alterations in genes responsible for homeostasis, cell regeneration and microbial interaction (188). These changes might be connected to the altered DNA methylation patterns observed in IBD patients (189).
Although the exact mechanisms are not understood, even in remission UC is associated with disruptions in the mucus system. This potentially eases relapses or disease onset.

7.6.3 Mucus in Crohn's Disease

In Crohn's disease, the mucus system is impacted differently. Early data suggested an increased thickness of the mucus barrier compared to controls (173). However, more recent studies comment on these findings and indicate that the thickness is not significantly different from healthy controls (175). On the other hand, a meta-analysis of studies on mucin expression in CD found a 34% reduction in total mucin expression (190). Loss of mucin expression is most prominent in ulcers with a complete disappearance of MUC2. Tissue surrounding ulcers exhibits ectopic expression of the gastric mucins MUC5AC and MUC6, suggesting a regenerative process. In healthy ileal mucosa, MUC2 levels are comparable to those in healthy controls (191).
The mucus characteristics in CD vary significantly according to the discontinuous inflammation pattern of the disease.
Goblet cell counts are reduced in CD, though not as drastically as in UC (182). But interestingly, in severe CD, goblet cell hyperplasia is observed in some patients. This includes various morphological phases of goblet cells, indicating high cell turnover (177).

And indeed, GC differentiation factors KLF4 and Hath1 are upregulated in active CD but not in UC, despite similar levels of inflammation (182).
In CD, extensive mucosal damage aligns with protective mucus-inducing countermeasures.
However, the mucins produced exhibit defects similar to those seen in UC. The polysaccharide chain length are reported to be shortened up to 50% (185) and mucins are weakly sulfated (177).

7.7 Akkermansia muciniphila

*Akkermansia muciniphila (*AM) is a commensal bacterium abundantly present in the human intestinal tract, comprising up to 1-4% of the bacterial population in the colon (192). Colonization occurs within the first year after birth, and its abundance decreases in elderly individuals (193). AM has recently gained interest due to its inverse correlation with numerous diseases like metabolic syndrome and inflammatory bowel disease (194). AM is considered a next generation probiotic because therapeutic interventions in mice have shown promising results.
AM is a non-motile, gram-negative bacterium of the Verrucomicrobia phylum. It does not form spores and is sensitive to oxygen (195). AM was initially classified as strictly anaerobic, but further studies have confirmed that AM can live under micro-aerated conditions and even increases its growth rate (196). Due to its oxygen reduction capacities AM tolerates traces of oxygen and thus enables a close association with the host epithelium (143).
Thorough analysis of AM's enzyme repertoire indicates its capability to metabolize a range of sugars including glucose, fucose, N-acetylglucosamine, and N-acetylgalactosamine. But when AM is grown on mucin it reaches its full potential, increasing the transport and metabolism of all these sugars while improving its growth rate (197). Mucin glycoproteins not only provide these essential sugars but also a protein backbone rich in threonine, which AM depends on. Even when AM is grown in mucus only, it is able to satisfy its needs for nitrogen, carbon, and energy. AM is highly adapted to the mucosal habitat. Mucin degradation in turn yields SCFAs for the host (195).

7.7.1 Bacteria shape their Habitat: Induction of Mucins

The abundance of bacteria in the gastrointestinal tract increases distally. In parallel, the mucus layer thickens to protect against the increasing bacterial load. This is reflected in the intestinal cell composition, with goblet cells making up 4% of the duodenum's cells and 16% in the distal colon (198). Findings in germ-free mice suggest that bacterial presence induces mucus production. Most data support a positive feedback loop between host mucus and microbiota (199).
In germ-free mice GC counts are reduced, the mucus layer is thinned and penetrable to beads the size of bacteria. Similar observations are made in Specific pathogen-free-mice (SPF mice) after antibiotic treatment. Establishing minimal microbial flora in germ-free mice restores mucus thickness, barrier capabilities, glycosylation patterns, and coating of fecal pellets, but this process takes six weeks (142, 200).
Many commensal bacteria shape their habitat by inducing their own separation and by stimulating MUC2 expression in proximal colon goblet cells. An interesting study has uncovered different glycosylation profiles of mucin proteins which correspond to their respective origin. For example, the mucus layer coating fecal pellets originates in proximal colon goblet cells. And interestingly it is exactly this type of mucin that is induced by bacteria. Bacteria thus stimulate their own encapsulation which preserves their habitat (142).
Microbial presence not only shapes host mucus production, this relationship can also be reversed. Inducing mucus production leads to changes in microbial composition, including an increased abundance of the mucus specialist AM (201). When the host cannot respond accordingly, for example, because of deficient O-glycosylation, beneficial commensals such as AM and *B. thetaiotaomicron* are depleted. This host-microbe cross-talk achieves coexistence and homeostasis. Inflammation is not only detrimental for the host but also destroys the commensals' habitat, resulting in changes in microbial composition, including a reduction of mucosal specialists like AM (69).
Even though unspecific bacterial molecules like LPS and peptidoglycan are sufficient to restore an adequate mucus barrier in germ-free mice (202), not all microbes stimulate the mucus barrier to the same extent. An interesting study compared SPF-mice of different housings and found that their housing is

associated with their mucus properties. Mice of one housing exhibited a thinner mucus layer, which was penetrable but the mucus barrier of the other housing was healthy. These differences were connected to their microbial composition which is broadly shared in a housing. Interestingly, the microbial composition associated with penetrable mucus, such as increases in proteobacteria, is comparable with observations in IBD patients (203). Transferring these bacteria to germ-free mice replicates the according mucus properties.
In obese mice and mice fed a high-fat, low-fiber diet, a disrupted mucus barrier with increased permeability and reduced MUC2 is present. Differences in microbial composition, including reduced AM, can be observed after only 3-7 days. Treatment with AM is able to reverse these mucus characteristics, suggesting a causal role of the microbiota (204, 205).
A lack of dietary fiber leads, as expected, to reduced SCFA levels. SCFAs are known to induce colonic mucin gene expression (206) but, on the other hand, have been shown to reduce mucus thickness (207). These results suggest that there are likely other mechanisms at play that lead to microbiota-induced mucus enhancement.
Troll, et al. demonstrated that intact Notch signaling is required for microbiota to restore goblet cell counts in germ-free zebrafish. Notch signaling controls differentiation into absorptive or secretory cells. Microbiota promote goblet cell differentiation via Myd88-signaling and consequently Notch inhibition (208).
As summarized above, commensals and their membrane vesicles tend to increase the regulatory cytokine IL-10, which is of most importance for intestinal homeostasis and tolerance towards commensals. IL-10 defective mice are used as model of IBD. Interestingly, IL-10 also governs mucin production by upregulating genes for mucin processing and folding, thereby maintaining proper mucin secretion and alleviating ER stress (209). ER-stress impairs GCs in their secretory properties (210).
Stimulation of autophagy also promotes a robust mucus layer by reducing ER-stress and consequently protecting from DSS-colitis (201). This effect is dependent on microbial presence and NOD2 as the receiving receptor.

7.7.1.1 *Akkermansia muciniphila* lives up to its Name

AM stands out in most studies investigating associations between microbiome and host mucus characteristics. Interestingly, studies far away from IBD have recently contributed to our understanding of AM. Metformin is used to treat type 2 diabetes mellitus (DM2), which is characterized by a desensitization of the insulin receptor. It was unknown how Metformin enhances insulin receptor sensitivity until this effect was connected to AM. Metformin induces changes in the intestinal microbiome, also increasing the abundance of AM. DM2 and decreased insulin sensitivity is likely caused by low-grade inflammation in visceral adipose tissue. AM enhances the intestinal barrier function and thus dampens inflammatory responses to microbial influx, ultimately improving the glycemic profile (211). Another study in mice confirmed these findings. Metformin treatment not only increases AM abundance but is also associated with higher MUC2 expression and goblet cell counts. Metformin was even able to alleviate DSS-induced colitis in mice. To confirm that Metformin relies on microbial changes, antibiotic treatment was shown to reverse the effects (212).

Other studies have shown that AM-prebiotics lead to upregulated MUC2, tight junction protein expression, increased mucus thickness, higher goblet cell counts and reduced age-associated intestinal permeability (213-215). The mice treated with AM increase goblet cell differentiation by upregulating transcription factors such as Dll1, Math1, and Spdef1 (216).

Despite its mucolytic properties, an increased abundance of AM is not associated with decreased mucus thickness. The opposite is the case, the mucus barrier is even reinforced.

7.8 Dysbiosis in Inflammatory Bowel Disease

The microbiome is a balanced ecosystem with an immense variety of microbes. Several diseases are linked to an altered gut microbiome. Accordingly, these alterations have been investigated as possible causes of disease. Dysbiosis means a disrupted microbial composition. It is measured by determining deviations from average healthy microbiomes. An example of this approach is the Bray-Curtis dissimilarity, which can be used at different taxonomic levels. The composition is considered dysbiotic when deviations from the healthy microbiome exceed a

certain threshold. Another common parameter of microbial composition is the diversity of different present species, known as α-diversity.
The true diversity of the microbiome has been uncovered via metagenomic studies. While these methods gather enormous amounts of molecular data, they also unravel varying results.
The most evident finding of studies on the gut microbiome is that the microbial composition of humans is highly individual. Even though the composition is influenced by several factors like health state, medication and diet, inter-individual variation among healthy individuals surpasses the impact of these other variables. This is why longitudinal studies with continuous follow-ups are more powerful than single cross-sectional studies and an appropriate sample size is essential.
Microbiome research has focused on the identification of bacteria enriched or depleted in disease states as a way of understanding their possible role in disease pathophysiology. The microbial disturbances in IBD are often described as a 'loss of beneficial microbes, rise of pathogenic microbes'. But it is important to note that we still do not have a definition of a 'healthy' microbiome and may never have. The majority of bacteria are simply neither 'good' nor 'bad' for their hosts. The term 'pathobiont' illustrates this: Microbiota can be beneficial in some circumstances and harmful in others (217). Classifying bacteria in buckets will never fully capture the complex reality. When the intestinal barrier or tolerogenic immune responses are disrupted, all bacteria will cause intestinal inflammation. Germ-free mice illustrate this concept: GF IBD-mouse models do not work properly very often, since there are no bacteria to trigger inflammatory responses. Therefore, IBD is characterized by a disrupted interplay between the host and microbiota.
But the microbial composition does matter, as it significantly influences the severity of colitis (218). Fecal bacteria from patients with UC, including patients in remission, induce a stronger inflammatory response compared to those from healthy controls (219).
While the microbial composition of patients with IBD differs from that of healthy controls, dysbiosis in IBD is also highly individual. Microbial variations that can manifest in different directions (220). A common feature across IBD microbiome phenotypes is decreased diversity (221). The changes in richness are reported to be more pronounced in CD compared to UC (222). IBD patients also experience

greater intra-individual fluctuations in microbiome composition over extended periods, indicating less microbial stability (223, 224).
At the phylum level, imbalances have been observed, though findings can be somewhat contradictory. In CD, facultative anaerobes like *Escherichia coli* bloom at the expense of obligate anaerobes (223, 225). Increased oxidative stress during inflammation may facilitate the migration of oxygen-tolerant microbes from the oral cavity to the intestine (226). Additionally, microbes considered opportunistic pathogens, including *Streptococcus*, *Burkholderia* and *Actinetobacter*, are enriched (222). At the family level, Lachnospiraceae and Ruminococcaceae are reduced (225). Generally, disturbances in composition are more pronounced in active disease (226). Compared to CD, the compositional changes in UC are more nuanced. In UC, obligate anaerobes are enriched and common findings of CD-Dysbiosis are not reproducible (222).
Several commensal beneficial species are altered in IBD, potentially exacerbating the disease. For example, *Alistipes putredinis* is depleted in IBD. This species normally prevents the overgrowth of Candida, which can contribute to inflammation by inducing Th-17 immune responses. Also, several SCFA producers including *Faecalibacterium prausnitzii* and *Ruminococcus bromii* are reduced in IBD (227). Metabolomic studies confirm a loss of SCFAs in IBD (223).
Under physiological conditions, the intestinal bacteria are well connected and assemble in clusters to complement each other. Bacteria enriched in IBD were shown to be absent from these networks of an established microbiome. These bacteria are not functionally integrated into the microbial structure (228).
These changes in microbial composition impact the host by affecting its gene expression. Bacteria control the activity of enzymes responsible for DNA-acetylation, initiate alternative splicing processes and directly regulate signaling cascades to change the activity of signaling pathways (229). In IBD, several host genes responsible for host-microbe interactions are differentially expressed (223). And indeed multi-omic studies have shown that alterations in host gene expression can be linked to changes in microbial composition, supporting this concept (230).
But assessing the genomic presence of microbes alone does not provide a complete picture of their functional roles. Investigating the transcriptional activity of

the gut microbiome allows this. Metatranscriptomic studies have revealed that functionality many times does not correspond to metagenomic data: some microbes may be genomically present and show low transcriptional activity, others may have greater functional consequences than expected from their abundance. Longitudinal studies on IBD have shown that changes in bacterial gene expression can be detected even when genomic abundance remains unchanged (231). This introduces the concept of “functional dysbiosis”. The microbiome can be disturbed functionally without changes in abundance of species. In IBD changes in microbial metabolome are more pronounced and sensitive than changes in microbial presence (223).

Since dysbiosis can also result from inflammation, its causality in disease development is not clear. Studies consistently show that microbial disturbances in IBD are closely connected to the disease state. Diversity and typical shifts in species are most prominently observed during active disease flares or unstable disease with short remission. Patients in long-term remission on the other hand exhibit a microbiome comparable to that of healthy controls (232). Inflammation, disease and dysbiosis are intertwined and not separable. The dilemma of which came first, the chicken or the egg, remains. However, this question might be missing the point because a vicious cycle results. Secondary phenomena can sustain this cycle even without being the root cause. A large body of evidence suggests that the composition of the microbiome plays an important role in IBD pathophysiology. This concept is confirmed by microbiome-related therapies, which have been shown to be effective in IBD.

7.8.1 *Akkermansia muciniphila*: A Key Player in IBD Pathophysiology

Akkermansia muciniphila is frequently implicated with IBD and provides further insights into the interplay of host and microbiome in IBD. Lower abundance of AM is commonly reported in both IBD entities, particularly during active disease phases. During remission, levels tend to return close to healthy controls (233-235). However, some studies report reduced AM levels even in remission, especially in Crohn's disease (236, 237). Generally, AM is strongly inversely associated with

inflammation, there is a correlation between low abundance of AM and higher inflammatory scores (238). One reason for this might be that inflammation alters the glycosylation and sulfation of mucin. Glycosylation in turn shapes the composition of the gut microbiome by selecting for matching inhabitants able to process this type of mucus. AM, for example, expresses sulfatases and thus selects its habitat based on the rate of mucin sulfation. Altered mucus characteristics in IBD might be the reason for the diminished levels of AM (235). Since other bacterial species rely on mucolytic bacteria to provide glycan fragments, changes in mucins also impact the microbiome as a whole.
When AM is administered to mice with DSS-colitis, it is able to restore microbial balance by promoting other species of the mucosal niche, illustrating its microbiome-governing effect (172, 239).
CD patients in remission were compared with healthy controls from the same environment. Dysbiosis and reduced amounts of AM are shown to persist even in remission (237). Additionally, AM is also reduced in non-inflamed colon tissue in UC patients (172), suggesting it precedes inflammation and is not solely a result of inflammation. Further longitudinal studies show that AM abundance correlates with sustained remission, and patients not achieving remission lack AM abundance (232, 240).
Studies on fecal microbiota transplantation (FMT) have been conducted in UC and confirm AM's role in dysbiosis. Donor stool rich in AM results in better treatment responses in receiving patients (241).

7.9 Hypothesis

BMVs are major effector particles responsible for many of the host's reactions to bacterial presence. Indeed, several benefits provided by AM can be replicated using AM-derived OMVs only. AM is known to reinforce epithelial barrier integrity and promote tolerogenic immune responses. These effects are now linked to its OMVs, which even alleviate disease severity in IBD-mouse models.
AM is also associated with enhancing the mucus barrier and goblet cell function, but there are no studies examining whether AM-derived OMVs contribute to this effect. In IBD observations match this association. The abundance of AM is lower and the mucus barrier is impaired. Understanding how AM induces mucus could

provide valuable insights for AM-based therapeutic strategies for IBDs.
Escherichia coli Nissle is another beneficial commensal associated with a functional host barrier and its OMVs alleviate disease severity in DSS-mice. Interestingly this was accompanied by a restoration of Trefoil Factor 3 (TFF-3), a crucial secretion product of goblet cells and a marker of mucosal integrity. Since TFF-3 is associated with goblet cell abundance, this provides further speculation of OMVs mediating the mucus system.
But these BMVs of commensal origin are known to generally reduce inflammation, which contributes to restorations in goblet cells. This illustrates that an understanding of molecular mechanisms at play is essential to better capture these host-microbe interactions (242).
The aim of this study is to investigate whether AM-derived OMVs mediate the mucus system. We focused on two key aspects: Do AM-OMVs induce goblet cell differentiation? Do AM-OMVs increase mucin expression?

7.10 Experimental Approach

We combined MUC2 quantification by flow cytometry with gene expression data from RT-qPCR. Flow cytometry was used to assess whether OMV treatment affects the ratio of MUC2 positive cells or increases the number of intracellular MUC2 per cell. Since expression of MUC2 defines a goblet cell, flow cytometry can provide insights on the fraction of cells which can be classified as such. In addition, quantitative polymerase chain reaction (RT-qPCR) was employed to measure the expression levels of different mucin proteins and goblet cell differentiation factors at the mRNA level.
Intestinal cell lines were used to explore their response to OMVs. The cell line LS174T is derived from a colorectal adenocarcinoma and consists of cells with a goblet cell phenotype with mucinous granules (243). Its mucin profile matches the expression in the colon since it predominantly expresses MUC2. Under physiological conditions, only goblet cells will stain positive for MUC2. HT29 is another cell line derived from a different patient with colorectal adenocarcinoma. It also grows in epithelial monolayers and expresses MUC2 (243). When treated with methotrexate (MTX), HT29 differentiate into a subpopulation with increased mucus-secreting properties (244). HT29-MTX predominantly expresses the gastric

mucin MUC5AC instead of MUC2. This mucin profile is valuable for different reasons. It provides insights whether bacteria or their BMVs can promote colon-like mucin expression or initiate regenerative processes since MUC5AC can be observed in colon issue of IBD patients, where its role in the regeneration of the mucosa is discussed. Also, HT-29-MTX cells might be better suited for the investigation of differentiation since, compared to LS174T, not every cell exhibits goblet cell characteristics.
HeLa and THP-1 cells do not express gastrointestinal mucins and were used as negative controls for the experimental setup.

We used OMVs from *Fusobacterium nucleatum* (FN) as experimental controls for experiments with AM-OMVs. FN is commonly found in the oral cavity, where it contributes to the development of periodontitis through biofilm production and the induction of inflammatory responses (245). In various diseases, particularly those involving colonic inflammation, oral microbiota have been shown to migrate to other sites, including the bowel (246). FN is not typically a member of the healthy gut microbiota (247) and associated with IBD and other GI disorders (248). Cell attachment and invasion contribute to its virulence. In microbial communities it also enables the invasion of naturally non-invasive species (249). The abundance of *Fusobacteriaceae* correlates with active disease in CD patients (250), making it a suitable comparison to the beneficial gut specialist AM. Also, FN is not associated with the mucus system and produces its own biofilm. Certain inherent components of OMVs, such as LPS, are known to influence mucus production. Therefore, comparism to FN helps identifing potential additional specific effects carried out by AM-OMVs.

7.10.1 Genes of Interest

AGR2 is an ER protein involved in the unfolded protein response in secretory epithelial cells. *AGR2* knockout mice (KO) exhibit increased ER stress and disrupted MUC2 synthesis and secretion, ultimately resulting in colitis (251). In affected mice, goblet cells (GC) can not be identified morphologically or via Alcian Blue staining. In zebrafish, defects in AGR2 result in a disrupted maturation of GCs (245), but mice do not require AGR2 for GC differentiation. In humans,

polymorphisms in the *AGR2* gene are known to affect the risk of developing IBD (252). GCs require AGR2 to properly process and secrete MUC2, but the mechanisms remain unclear. Mostly an involvement in translation or post-transcriptional processing are under discussion (253).
KLF4 is a transcription factor that regulates the differentiation of GCs. *KLF4*$^{-/-}$ mice do not properly develop GCs and also display abnormal Alician Blue staining and expression of MUC2. GCs are diminished, and characteristic GC morphology and ultrastructural features cannot be observed, although other epithelial lineages remain present (254). *KLF4* is regulated by Notch signaling as activity of Notch inhibits KLF4 expression (255). Mice treated with γ-secretase inhibitors show an upregulation *of KLF4*. This results from increased proteolytic processing of Notch since Notch is a γ-secretase substrate. The upregulation of *KLF4* then promotes GC differentiation in proliferating crypt cells. Additionally, adenoma cells in mice with APC mutations also convert into goblet cells upon γ-secretase inhibitor treatment (256).
To gain a more comprehensive understanding, we included *MUC5AC* and *MUC1* in addition to *MUC2*. Above we have introduced how bacterial presence influences mucus characteristics and the epithelial lineage. These features differ between the upper gastrointestinal tract and the colon, suggesting bacterial influence. Furthermore, during colonic inflammation and IBD, the gastric mucin MUC5AC is also expressed in goblet cells alongside MUC2 (257). Paneth cell metaplasia is another feature of the upper gastrointestinal tract and occurs in IBD.
Simulating bacterial presence with an OMV treatment could also answer the question whether bacteria are able to reverse these upper gastrointestinal tract features to a colon-like pattern. We additionally included the transmembrane mucin MUC1. Its role in host-microbiota interaction and barrier function is largely unknown.

8 Material and Methods

8.1 Bacterial Cell Culture and Vesicle Isolation

Akkermansia muciniphila type strain (ATCC, BAA-835) was obtained from LGC Standards (Wesel, Germany) and cultured in a brain heart infusion medium with 0.5% porcine mucin (Sigma-Aldrich, Darmstadt Germany) and 0.5 g/L L-Cysteine (Sigma-Aldrich) in an anaerobic chamber for 7 days at 37 °C.
OMV isolation was performed by Schild's lab (University Graz), generously contributing their expertise on bacterial membrane vesicles. The isolation procedure was conducted as follows: In the late exponential growth phase, bacterial cells were pelleted by centrifugation at 6,000 and 9000 × g for 15 minutes at 4°C. The supernatant containing the vesicles was collected and then filtrated through 0.45-μm and 0.22-μm pore size filters to filter residual cell debris. To verify the absence of bacterial cells, 1 milliliter of the filtrate was cultured on an LB agar plate and incubated overnight. To prevent protein degradation, a protease inhibitor (complete EDTA-free protease inhibitor cocktail, 1 tablet per liter of filtrate; Roche) was added to the filtrate before storage at -80°C.
The outer membrane vesicles (OMVs) in the supernatant were isolated by ultracentrifugation at 150,000 × g for 4 hours at 4°C and resuspended in saline to achieve a concentration 1,000 times higher than the initial filter-sterilized supernatant. Protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories), and values were normalized to the optical density of the original culture (258).

8.2 Cell Lines and Cell Culture

Cell lines HT-29, HT-29-MTX, Hela, THP-1 and LS174T and their respective cell culture media (DMEM, RPMI-1640, EMEM) were obtained from Sigma-Aldrich and cultured at 37°C, 5% CO_2, atmospheric O_2.
Frozen cell suspensions were thawed, then the cytotoxic DMSO was deactivated with fetal bovine serum (FBS) and removed by centrifuging. Cells were initially seeded in medium with 20% FBS (Gibco), 1% penicillin/streptomycin (Gibco), and 1% GlutaMAX (Gibco). After two days, the FBS concentration was reduced and kept at 10%. Cell viability and confluency were monitored regularly under the

microscope. Sub-confluent cells (70%) were passaged. Here the cells were incubated for 6 minutes at 37°C with 0.25% Trypsin-EDTA (Sigma-Aldrich), resuspended, and the trypsin was deactivated with medium and finally discarded after centrifugation. After automatic cell counting with a CASY TTT cell counter (OMNI Life Science, Bremen, Germany), cells were reseeded at the desired concentration.

8.3 Flow Cytometry

After detaching the cells with trypsin, they were centrifuged and resuspended in PBS to a concentration of 10^6 cells/mL. To 100 µL of the cell suspension, 100 µL of formaldehyde-fixation buffer (Invitrogen™ eBioscience Intracellular Fixation & Permeabilization Buffer Set) was added. The cells were incubated for 45 minutes, protected from light. Then intracellular staining was enabled using 1 mL of permeabilization buffer (Invitrogen™ eBioscience Intracellular Fixation & Permeabilization Buffer Set). Subsequently the cells were washed two times. Washing steps include the resuspension with 1 mL permeabilization buffer and centrifuging. Next, 200 µL of staining buffer, containing 0.5% bovine serum albumin (Thermo Scientific) and 2 mM EDTA (Sigma-Aldrich) in Dulbecco's phosphate-buffered saline (DPBS, Gibco), was added to block nonspecific binding sites. After that 1 µg of non-conjugated primary antibody (Mouse anti-MUC2 IgG, MA5-12345, Invitrogen) diluted in 100 µL permeabilization buffer was added. After 30 minutes of incubation, cells were again washed twice with permeabilization buffer. Now 1 µg of the secondary conjugated antibody (Goat anti-Mouse IgG, Alexa Fluor 488, Invitrogen) completed the staining. After incubation, cells were washed twice again. The pellet was then resuspended in 200 µL of staining buffer and analyzed by flow cytometry. Measurements were performed using a CytoFLEX LX flow cytometer (Beckman Coulter).

8.3.1 Data Analysis

Particularly LS174T sheds significant amounts of debris, which was excluded using appropriate gating in a forward versus side scatter plot (FSC vs. SSC). Doublets were excluded using a FSC-height vs. FSC-area plot (259). FlowJo

(v10.10.0) was used for data analysis, and the gating strategy is illustrated in Supplemental Figure 1. Since we did not observe distinct populations of MUC2-positive and MUC2-negative cells, the threshold for a MUC2-positive cell was set based on negative controls represented by cell lines that do not express MUC2. This threshold provides a useful reference and is represented in the graphs. However, since this threshold is arbitrary, all statistical calculations were made using the mean fluorescence intensity (MFI) value. For orientation, both the mean and median fluorescence intensities were calculated (Supplemental Figure 2). The MFI (mean) best represented the data, as the median did not capture changes in MUC2 expression that were visible in the graphical display. Since the median is susceptible to outliers, events outside the expected range were excluded by gating. The MFI data was visualized in boxplots by using datatab.de. The MFIs of control and treatment groups were compared using one-sided two-sample t-tests, assuming unequal variances, in SPSS Statistics 29. While the expression of MUC2 is not normally distributed in LS174T cells, the extrapolated MFI can be assumed to follow a normal distribution, allowing for the use of parametric tests. In treatment experiments, we always used the same cell line as untreated controls. We also observed that unstained controls were unaffected by DAPT and OMVs. Therefore, subtracting the MFI of unstained controls, which corrects for autofluorescence, was not necessary.

8.3.2 Addressing potential unspecific Staining

Initially, biological negative control cells (THP-1 and HeLa, which do not express MUC2) and unstained controls were used to refine and adapt the protocol, by comparing them to our cell lines of interest. Possible unspecific binding of the antibody was addressed by comparing the standard protocol to additional blocking buffers containing 5% bovine serum albumin (BSA, Gibco) and 5% FBS (Gibco). Additional washing steps were included (three washes instead of two) before and after staining. To further reduce nonspecific binding, the indirect staining protocol was modified to direct staining with a conjugated primary antibody (Anti-MUC2, Alexa Fluor 647, Novus Biologicals).

8.3.3 Evaluating a Positive Control

In treatment experiments, cells were generally seeded to reach confluency during the observed time frame, as the differentiation process is essential to our research questions. For each cell line or treatment of interest, three biological replicates were performed. DAPT is a γ-secretase inhibitor known to induce goblet cell differentiation by disinhibiting Notch signaling. DAPT has been shown to induce goblet cell differentiation in Lgr5+ stem cell-derived monolayer cultures (260), but also inhibits cell proliferation. In the evaluation experiment, medium with DAPT (10 µM in DMSO, as previously described (260)) was changed on days 2, 4, and 6 after seeding. Cells were harvested on day 7 and compared to cells cultured with medium without DAPT. HT-29-MTX and LS174T cells were evaluated in this experiment.

8.3.4 OMV Treatment

LS174T cells were chosen for the OMV treatment based on previous experiments. We captured three different time points after the OMV treatment. For each time point and treatment (500 ng/mL OMVs, 10 µM DAPT, or control with regular medium), three biological replicates were performed. Cells were seeded on day 0 with the corresponding treatment, and the medium with according treatment was changed on days 2, 4, and 6. Cells were collected and transferred to flow cytometry on days 1, 4, and 8.

8.4 RT-qPCR

The OMV treatment was conducted as a timeline experiment with five time points following a single OMV exposure (4 h, 8 h, 16 h, 24 h, and 32 h). Cells were seeded to surpass their pre-confluent state within the observed time frame. We used OMV concentrations used previously in other studies (113), namely 500 ng/mL and 100 ng/mL. We additionally included three untreated controls per timepoint. In total, four separate wells of cells for each time point and treatment, were examined and plotted in the graph.

8.4.1 Protocol and Data Analysis

The cell monolayer was washed once with PBS to remove excess debris. To starte the RNA-isolation 800 µL of TRIzol (Invitrogen) was added to each 4 cm² well to detach and resuspend the cells. The suspension was vortexed and snap-frozen in liquid nitrogen to lyse the cells. After thawing, 200 µL of chloroform was added and vortexed. The samples were centrifuged at 12,000 x g for 15 minutes. Now the upper RNA-containing aqueous phase was pipetted off without disturbing the interphase and transferred to new tubes. Then 500 µL of isopropanol was added and the samples were centrifuged at 12,000 x g for another 10 minutes, which finally provided the RNA pellet. The supernatant was discarded carefully with a pipette and the RNA pellet was resuspended and washed with 1 mL of 75% ethanol. After discarding the supernatant the pellet was air-dried and finally resuspended in RNase-free water. The RNA concentration was measured with a NanoDrop instrument (Thermo Fisher Scientific) and equalized across samples.

To degrade any remaining DNA, DNase treatment was performed. For this purpose, each sample was incubated for 30 minutes at 37°C with 1 µL of DNase (Promega) per µg of total RNA. After that 1 µL of DNase Stop Solution (Promega) was added and the treatment was completed with 10 minutes of incubation at 65°C. Hereafter, the High-Capacity cDNA Reverse Transcription kit Applied Biosystems was used to produce cDNA. In a reaction volume of 20 µL we combined 1000 ng of RNA and 10 µL master mix as specified by the supplier (including buffer, random primers, dNTP mix, reverse transcriptase (MultiScribe), and RNase inhibitor (Promega RNasin)). The reactions were conducted in a peqSTAR Thermocycler (Peqlab) with the following program: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. The resulting cDNA was diluted and stored at -80°C.
The next step was Real-Time Quantitative PCR (qPCR) using the SYBR Green Universal Master Mix (Applied Biosystems). Each qPCR reaction consisted of a total volume of 10 µL, containing 20 ng of cDNA and 6 µL of master mix combined with the respective forward and reverse primers. Primers were obtained from OriGene Technologies (Supplemental Figure 3). The expression of each gene of interest was analysed in technical triplicates. Based on the calculated number of

reactions, the wells were pipetted using the Biomek i7 automated workstation (Beckman Coulter) and amplified in a CFX96 PCR cycler (Bio-Rad) with the following protocol: 39 cycles of 15 seconds at 97°C and 1 minute at 60°C. Following amplification, a melt curve analysis and no-template controls (NTCs) were used to assess the specificity of the amplification process.
The data was transferred to Excel where the ΔΔCt method was used to calculate the respective gene expressions. Ct-values were first corrected for primer efficiency, which was previously determined by assesment of standard curves. Technical triplicates were averaged to obtain mean Ct-values. The ΔCt for each sample was then calculated by subtracting the Ct of the housekeeping gene *ACTB* from the Ct of the target gene. To calculate ΔΔCt, the averaged ΔCt of the control samples of each respective time point was subtracted from the ΔCt of treatment samples. Fold change was subsequently determined using the formula $2^{-\Delta\Delta Ct}$ and displayed in a boxplot created in GraphPad Prism (version 10.1.1).
Raw Ct-values and fold changes were not used for statistical calculations (261), as ΔCt-values are normally distributed and suit best for statistical analysis since they indirectly represent fold change and ΔΔCt by comparing to the controls. One-sided two-sample t-tests, assuming unequal variances, were performed in SPSS Statistics (version 29).

9 Results

9.1 Flow Cytometry struggles to distinguish Goblet Cells in intestinal Cell Lines

Flow cytometry was used to determine the amount of intracellular MUC2 per cell as surrogate for MUC2 production and to observe the ratio of MUC2 positive cells

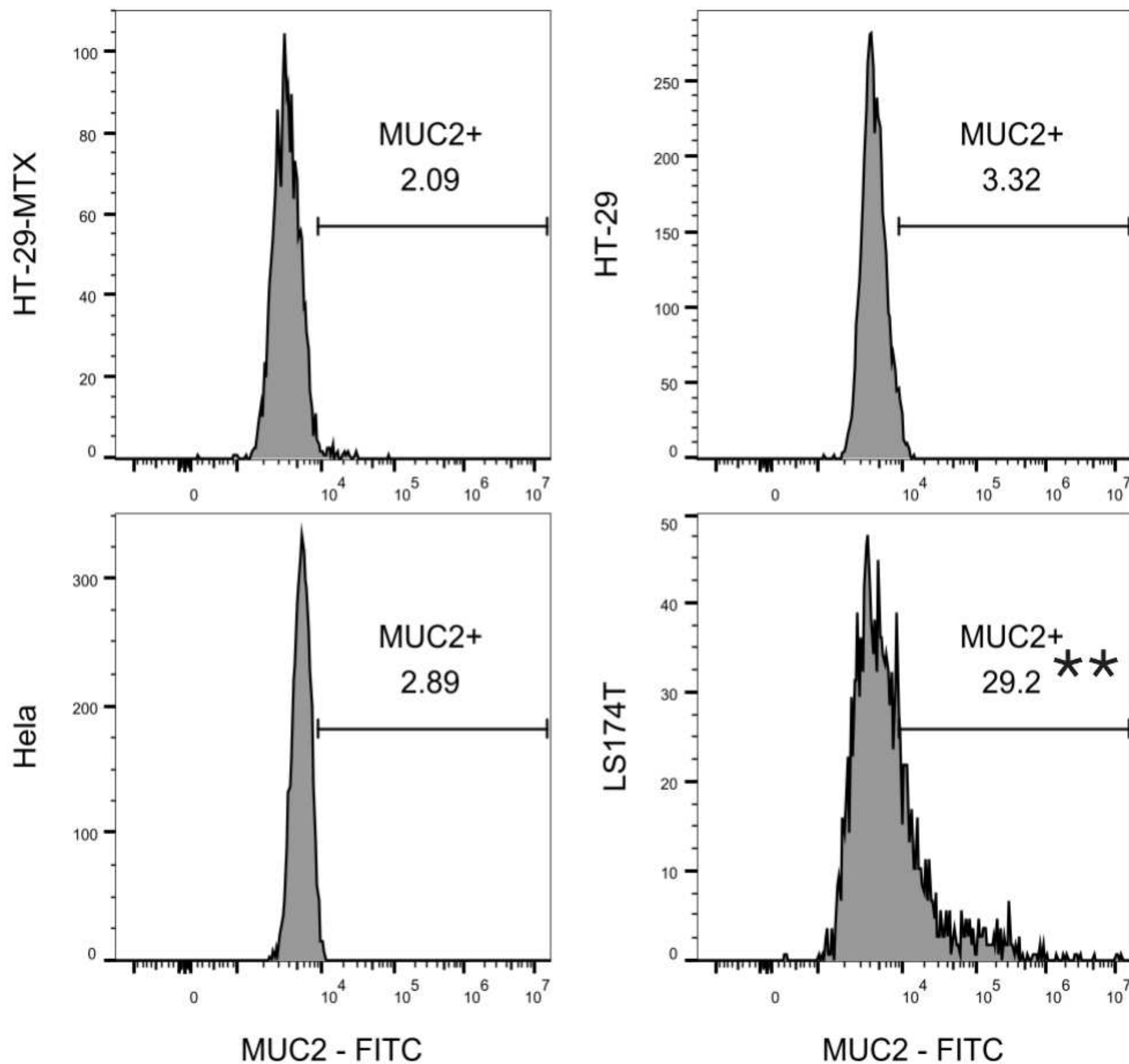


Figure 3: *MUC2 Expression in intestinal Cell Lines*

*Flow cytometry analysis of intestinal cell lines HT-29, HT-29-MTX and LS174T compared to non MUC2-expressing Hela (THP-1 not shown). Plots show their representative MUC2 fluorescence signal. Gating was applied to indicate the percentage of cells exceeding an arbitrary threshold of MUC2 fluorescence intensity. Statistical significance between intestinal cell lines and Hela was assessed by comparing their mean fluorescence intensities (MFI) using two-sample t-tests. **P<0,001.*

to MUC2 negative cells as an indicator of goblet cell abundance. In preliminary experiments, we tested intestinal cell lines known to express MUC2 (HT-29, HT-29-MTX, and LS174T) and non-MUC2-expressing lines (HeLa, THP-1). We noticed that only LS174T differ from negative control cell lines with increased intracellular MUC2 (a right-skewed distribution in Figure 3). LS174T feature homogeneous cells with goblet cell differentiation, as the distribution of MUC2 fluorescence intensity in LS174T represents a continuous spectrum, rather than two separate populations. Thus, appropriate gating in goblet cells and non-goblet cells to approximate the in vivo situation is not possible. Instead, the fraction of cells surpassing a certain MUC2 threshold was determined to define MUC2-positive cells. This threshold was oriented on the cell lines not expressing MUC2 but is still arbitrary.

On the other hand, the cell lines HT-29 and HT-29-MTX are known to express less MUC2. This was confirmed in flow cytometry, as both cell lines could not be distinguished from MUC2 negative control cell lines THP-1 and Hela by their MUC2 fluorescence. HT-29-MTX feature only a subpopulation of MUC2-expressing cells (244), but here we show that flow cytometry was not sufficient to identify this population.

To further explore the cell lines in flow cytometry, we introduced positive control treatments. DAPT has been shown to induce goblet cell differentiation in multiple studies (260, 262, 263), making it a promising candidate. HT-29 and LS174T were compared in their response to DAPT (10 µM). As shown in Figure 4, the ratio of LS174T cells expressing higher levels of MUC2 significantly increased and the mean fluorescence intensity (MFI) was significantly higher than in controls ($p< 0,01$). HT-29 cells did not react to the treatment. We also visually observed that DAPT-treated cells were less confluent, confirming its inhibitory effect on proliferation. Eventually, LS174T cells were selected to investigate the potential effects of AM-OMVs.

9.2 *Akkermanisa muciniphila* OMVs did not increase the Ratio of MUC2 Positive LS174T Cells

The OMV treatment was carried out as a timeline experiment. The cells were seeded sub-confluently to observe their differentiation under treatment. Over the

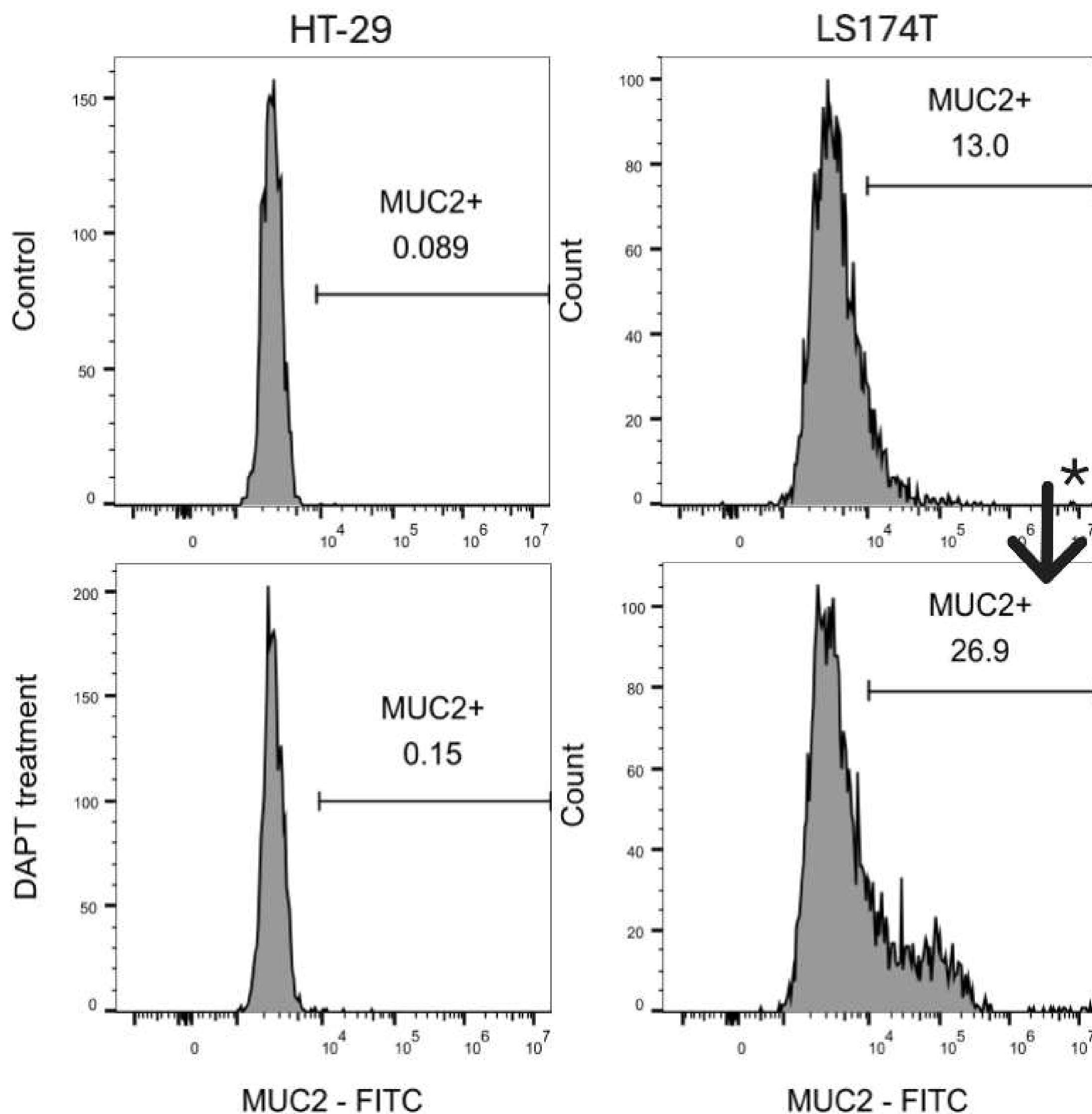


Figure 4: *DAPT Treatment in LS174T and HT-29 Cells.*

*Flow cytometry analysis of MUC2 expression in HT-29 and LS174T. Plots compare the representative MUC2-fluorescence of DAPT treatments and controls. Gating was applied to indicate the percentage of cells exceeding an arbitrary threshold of MUC2 fluorescence intensity. Statistical significance between treatment and control was assessed by comparing their mean fluorescence intensities (MFI) using two-sample t-tests and is indicated with arrows. *P<0,01.*

observed time frame, LS174T cells increased their MUC2 expression (Figure 5). This is an expected phenomenon of differentiation.

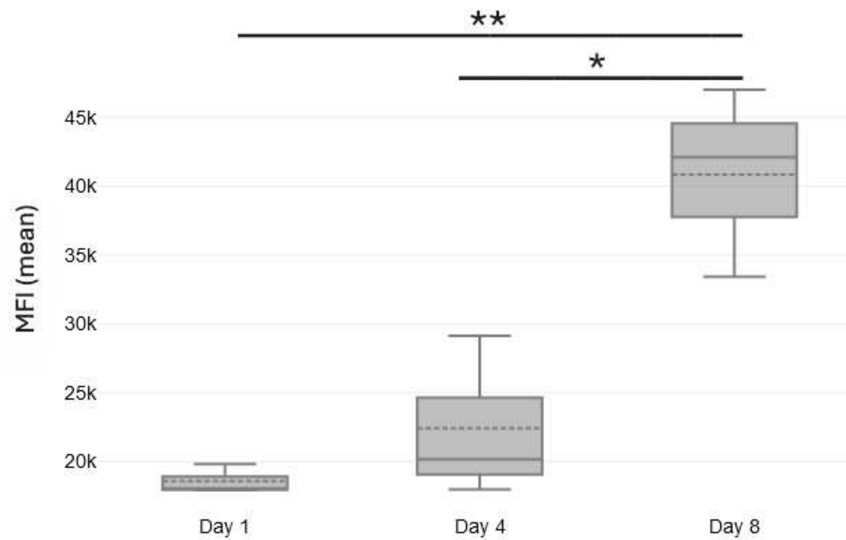


Figure 5: *Differentiation of LS174T Cells*

*Flow cytometry analysis of MUC2 expression in untreated control cells, comparing the MFI at each time point. Each boxplot consists of 3 separate wells of cells. Statistical significance between time points was assessed by comparing their mean fluorescence intensities (MFI) using two-sample t-tests. *P <0 ,01 **P <0,001*

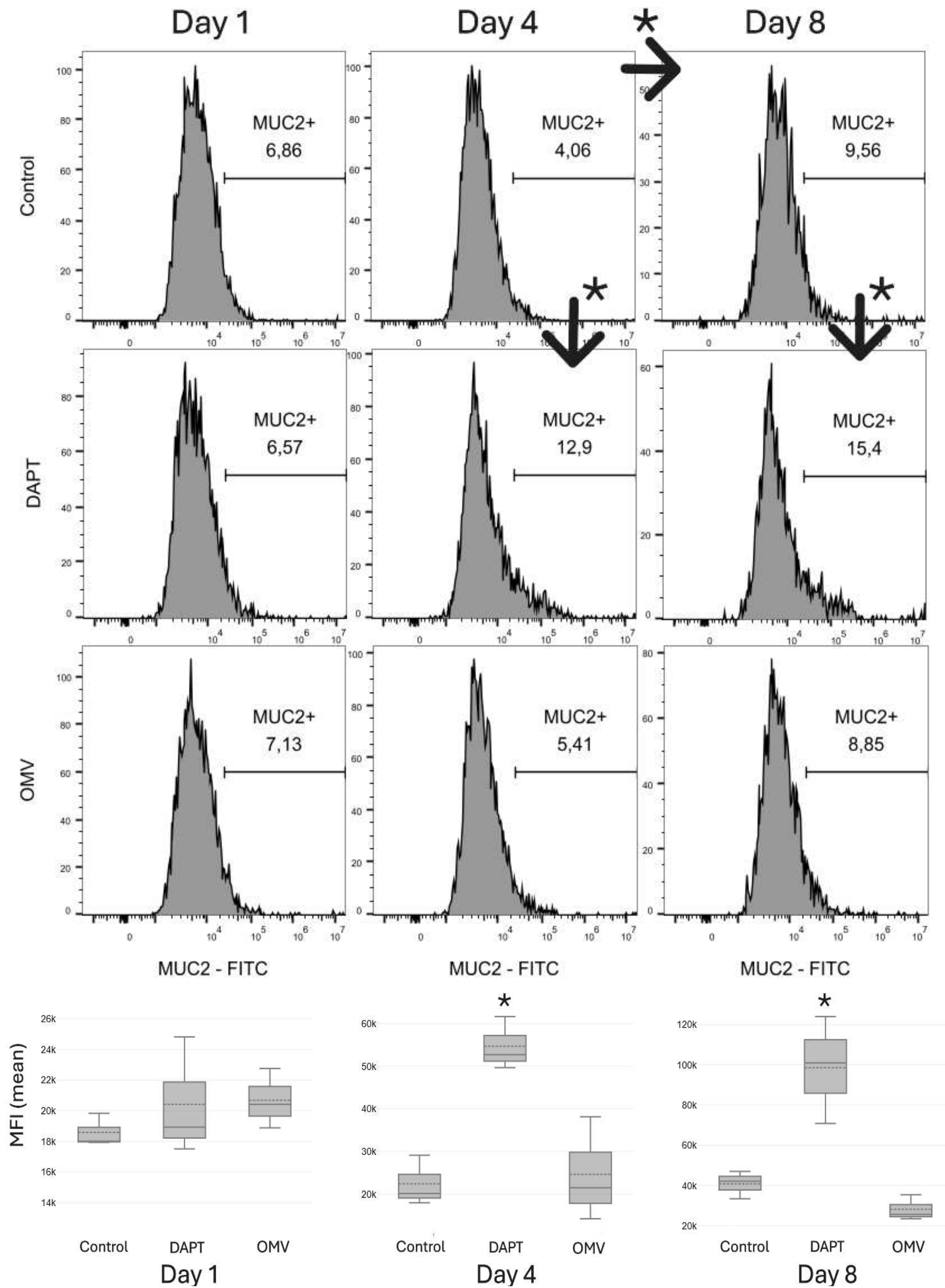


Figure 6: *OMV Treatment – Timeline Experiment*

Flow cytometry analysis of MUC2 expression in LS174T upon DAPT and OMV-treatment compared to controls on three time points. Statistically significant differences between time points or treatments are indicated with arrows. Significance was assessed by comparing their mean fluorescence intensities (MFI) using two-sample t-tests.
*Boxplots show the MFIs of each day and respective treatment. *P <0 ,01*

The treatment responses reveal that the influence of DAPT (10 µM) on goblet cell differentiation took longer than one day (Figure 6). DAPT influences Notch signaling and KLF4 to eventually induce goblet cell differentiation. This process is expected to take several days and only becomes measurable when cells differentiate. Interestingly, DAPT increased the mean fluorescence intensity on days 4 and 8, but did not affect the median fluorescence intensity (Appendix 3) on these days. This suggests that DAPT did not induce MUC2 in every LS174T cell. Rather, cells which already have a higher baseline of MUC2 expression appeared to respond to the treatment.
In contrast, treatment with AM-OMVs (500 ng/mL) did not lead to an increase in intracellular MUC2 levels or in the proportion of MUC2-positive cells.

9.3 *Akkermansia muciniphila* OMVs did not induce Goblet cell Differentiation or Mucins in HT-29-MTX

In flow cytometry, we did not detect changes in intracellular MUC2 levels upon AM-OMV treatment. Even though MUC2 protein levels seem to be unchanged, AM-OMVs might still influence the expression of mucin genes or genes related to goblet cell function and differentiation. For this reason, RT-qPCR was conducted to explore more subtle changes in the mucin system. In addition to *MUC2*, other mucin genes were analyzed to determine whether OMV treatment could shift the expression profile towards a colon-like mucin pattern. The expression of *AGR2* which is essential for goblet cell functionality and goblet cell differentiation factor *KLF4* have also been investigated. As an experimental control OMVs of the tumorigenic intestinal bacterium *Fusobacterium nucleatum* (FN) were included. HT-29-MTX cells were chosen for this approach. They may provide more insights in differentiation processes than LS174T cells, which are already differentiated into goblet cells. Again, the experiments were conducted as a timeline experiment to capture expression kinetics.
Firstly, we observed similar behavior to that seen in LS174T cells. HT-29-MTX cells showed a continuous increase in *MUC2* expression over time as they differentiated, indicated by steadily rising Ct values for *MUC2*. In OMV-treated cells, no gene of interest showed significant differences ($p<0,05$) to untreated control samples at any given time point. Although the relative mRNA expression

exhibited hints of induction, these appeared random and were not dose-dependent, suggesting they are likely due to biological fluctuation.

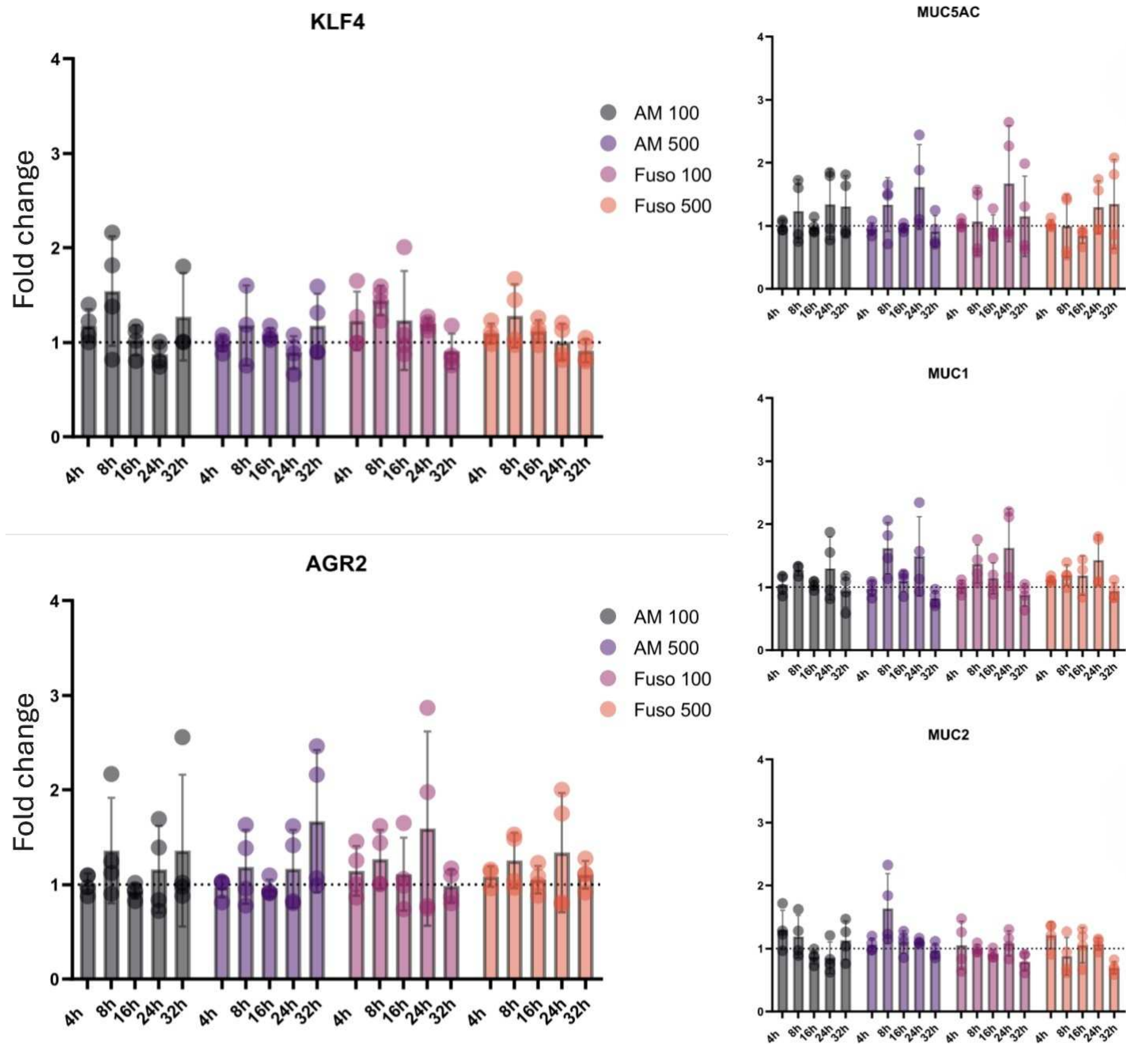


Figure 7: *mRNA Expression of MUC1/MUC2/MUC5AC and Goblet Cell differentiation Factors KLF4 and AGR2 in HT-29-MTX following OMV Treatment*

Treatment with varying concentrations (500 and 100 ng/ml) of Akkermansia muciniphila or Fusobacterium nucleatum OMVs, respectively. Fold change was calculated using the 2–ΔΔCt method. Each dot represents an individual well of cells. Statistical significance was assessed by comparing the ΔCt values of treated samples with those of controls. No time point reached statistical significance with $P < 0.05$.

10 Discussion

Akkermansia muciniphila (AM) is a commensal bacterium which is specialized in metabolizing mucin. It lives in the mucosal nieche close to its host. Many studies have linked AM to the enhancement of the intestinal mucus barrier and immunomodulation. Some of these effects were now connected to its OMVs, which induce the expression of tight junction proteins and the differentiation of Tregs. These effects maintain homeostasis and are sufficient to alleviate colitis in mice. AM is commonly associated with healthy mucus characteristics, but there are no studies examining its OMVs in this context. Currently, only two studies have explored MUC2 expression and goblet cell abundance in DSS-colitis mouse models (113, 264). These studies reported that AM-OMVs restore goblet cell populations and MUC2. However, these findings could also be attributed to the anti-inflammatory effects of AM-OMV since inflammation is known to be associated with mucus depletion. Here why tried to gain further mechanistic insights to differentiate these observations. Understanding the underlying mechanisms is important for novel IBD treatments based on AM and its OMVs. MUC2 expression was determined with flow cytometry and not only serves as a surrogate of mucus production but also is a goblet cell marker. The endpoint of goblet cell differentiation could not be properly investigated with flow cytometry since LS174T cells generally are goblet cell differentiated and do not display subpopulations of MUC2 positive and negative cells. Accordingly, flow cytometry might be more sufficient to determine goblet cell counts in cells of in vivo tissue or primary cell culture. Measuring the amount of intracellular MUC2 protein was only sufficient in LS174T, but this parameter is only a snapshot of one fraction of the mucin machinery. Flow cytometry is not the method of choice to investigate mucin properties of mucus-producing gastrointestinal cell lines.
To further assess goblet cell differentiation and include mechanistic insights, mRNA of mucin genes and transcription factors implicated in mucin expression have been analyzed with RT-qPCR.
In all treatment experiments with OMVs of AM and FN, we found no significant changes in the observed parameters. Thus, our study did not provide evidence to support a direct role of AM-OMVs in modulating mucus characteristics. Further

research employing alternative methods is required to test the hypothesis that AM utilizes its OMVs to mediate its mucus-regulating effects.

10.1 Reducing a complex System

Our experimental approach represented a highly simplified model of the complex in vivo host-microbe interactions. The mucus layer properties were reduced to MUC2 expression and certain goblet cell transcription factors. As previously introduced, the mucin system and its regulation is highly complex. The expression of *MUC2* mRNA or intracellular MUC2 protein amounts cannot fully represent production or secretion levels. Other studies have shown that *MUC2* mRNA expression and actual mucin secretion are often not concordant (137). Post-transcriptional modifications, for instance, are essential for creating the structure of functional MUC2 and left out by our approach. We exclusively focussed on intracellular MUC2 and did not account for secretion. For example, a MUC2-specific ELISA could have been used to quantify MUC2 in cell culture supernatants. LPS is carried by OMVs and is a known stimulator of mucin secretion. This expected effect would have confirmed that the OMVs interacted with the cells.
But even when mucus secretion is equal, functional changes in its structure can lead to its dysfunction. Our study did not investigate functional differences in mucus properties. Parameters like mucus thickness and permeability, glycosylation profiles and proteomics are essential for a complete characterization. Bacteria were previously shown to particularly affect mucus permeablity.
An interesting study connected autophagy, ER stress and the mucus system and reported the difficulties in unraveling the interconnections in these systems. *Beclin-1* knock-in mice exhibit increased mucus secretion due to enhanced autophagy pathways. These mice show no observable changes in goblet cell granules, goblet cell number, mucin gene transcription, goblet cell transcription factors, or reactive oxygen species levels (201). In this case, increased mucus secretion was attributed to the alleviation of ER stress by an overactivation of autophagy. ER stress itself is associated with dysfunctional goblet cells (210), adding another layer to the regulation of goblet cells and the mucus barrier.

Our study examined only a small subset of these parameters within the mucus system. The system could still be affected in different pathways. BMVs were already shown to interact with various signaling pathways.
Another limitation of our approach was the reduction of the complex intestinal mucosa to intestinal epithelial cell lines. These cell lines are derived from tumors with an inherently impaired differentiation. They do not represent the distinct epithelial populations observed in vivo. This limitation was particularly evident in flow cytometry, where we were not able to distinguish goblet cells as a distinct subpopulation characterized by the presence of MUC2. Instead, all LS174T cells showed MUC2 expression in varying amounts. This is why MUC2 cannot be sufficiently used to characterize a gobelt cell and thus investigate goblet cell differentiation.

10.2 Experimental Approach

Our study represents a simplified experimental reduction of cellular responses and signaling mechanisms within the mucus system. Because of the inherent shortcomings of this approach, we are not able to prove the hypothesis that bacterial membrane vesicles modulate the host mucus system.
In flow cytometry, we generally did not observe the expected differences between MUC2-expressing cell lines and negative controls, which led us to suspect nonspecific staining in the negative controls. The MUC2 signal in LS174T could still be caused by specific cell characteristics leading to unspecific binding of the antibodies. As different cells vary in properties like granularity and size, they may also show differing levels of nonspecific staining. This is the reason why comparing different cell lines in this setup is technically incorrect. But it still provides valuable insights for refining the experimental setup. In OMV treatment experiments, untreated cells of the same cell line were used as controls. In troubleshooting experiments, we found that the secondary antibody was responsible for most of the nonspecific staining. MUC2 is a common intracellular protein which should not require signal amplification through indirect staining. As a result, we switched to a conjugated primary antibody. Refining our protocol did not change the general outcome with HT-29 and HT-29-MTX not deviating from negative controls.

Additionally, the use of OMVs in cell culture is a novel concept and not well-documented in literature. Designing the experiments and handling the OMVs lack standardization. Our static monolayer is coated with a mucus layer but misses intestinal motility and the transport of mucus. OMVs may be hindered in reaching the epithelial cells. Additionally, in vivo, AM resides in the mucus layer and produces vesicles close to the epithelium. These limitations are particularly relevant for LS174T cells since they produce large amounts of mucus and debris. Vesicle-cell interactions may be more difficult to be assessed than in other cell lines. Our chosen OMV treatment concentrations were consistent with previous studies using other cell lines. However, a recent study also used LS174T cells but enrolled their OMV treatment with concentrations 20 times higher than in our experiments (265). Another group treated HT-29 cells in concentrations 200 times higher (113). Monolayers with mucus production seem to require higher concentrations. Systematic concentration testing should have been done in this uncertain setup. Maybe with increased concentration there indeed will be measurable consequences. At the time of this study, there was no data on the combination of LS174T or HT-29-MTX cells and AM-OMVs. Positive control treatments like LPS and DAPT should have been included in RT-qPCR to better evaluate and troubleshoot our setup. We have no verification that the OMVs reached the cells and interacted with them. In theory, *Fusobacterium nucleatum* OMVs serve as an interesting experimental control, but only when the experimental setup is verified.

In the future alternative methods should be considered. A recent study had similar aims but used AM-OMVs on LS174T cells with indomethacin-induced mucus secretion dysfunction. This study demonstrated that AM-OMVs can restore mucus secretion by alleviating ER stress (266).

Primary cell culture provides a more accurate replication of the intestinal epithelium and would be suitable for studying goblet cell differentiation. These cultures preserve normal differentiation processes and distinct goblet cell subpopulations. Primary cultures derived from germ-free mice provide additional experimental benefits as they exhibit reduced goblet cell counts and a defective mucus layer. OMV treatment might reverse these defects caused by the absence of bacterial presence.

In IBD mouse models, AM-OMVs have been suggested to enhance mucus barrier properties. However, it remains unclear whether these effects are due to the reduction of inflammation. Aged mice could provide another mode with decreased mucus thickness increased mucus permeability (267). This could offer a more direct assessment of their effects on the mucus barrier, independently of inflammation.
The role of BMVs in modulating the intestinal mucus system is still unexplored. We have provided an initial glimpse with its limitations, further studies with different methods have to be employed.

10.3 Bacterial Membrane Vesicles as Key Players in IBD Pathogenesis

Even though the effects of BMVs on the mucus system remain unclear, they have been shown to carry out numerous other homeostasis-inducing outcomes. These effects on the host are sufficient to reduce disease severity in IBD mouse models. BMVs are mediators of microbiome-host interactions. Here, we propose a model demonstrating the role of BMVs in IBD pathophysiology. We suggest that disrupted host-microbe homeostasis may arise from impaired interactions at the BMV level. The communication system of BMVs maintaining homeostasis can break down on either the microbial (A) or host side (B) of the relationship.

10.3.1 A: Disrupted Influx of Bacterial Membrane Vesicles

As described above, the microbiome is disrupted in IBD, either in its composition or functional state. The significance of the microbiome in IBD is well established, as alterations to the microbiome can directly influence the disease severity. However, the distinct mechanisms of how the microbiome affects the disease are unknown. Recent studies suggest that many of the microbiome’s effects are at least partly mediated by BMVs. This may represent a mechanism through which dysbiosis exerts its effects on the host, contributing to the development or persistence of IBD.
Logically, an altered microbial community also results in a different BMV signal to the host. This was confirmed by metagenomic studies that observed that changes in microbial presence are associated with an altered BMV signal (31). An interesting study compared the gut microbiome and BMV composition of CD

patients with healthy controls. Bacterial richness is reduced is reduced in abundance and among the BMV fraction. But interestingly the microbial diversity is only reduced in the BMV composition. Here the activity of key species such as *Faecalibacterium prausnitzii* and *Ruminococcus* are reduced. The homeostasis-inducing effect of their membrane vesicles has been introduced before (31).
In another study, BMV composition during DSS-induced colitis in mice revealed more pronounced shifts in BMVs than in bacterial abundance, with reduced membrane vesicles from species such as *Bacteroides* and *Akkermansia* (106). This suggests that the BMV composition may be a more sensitive indicator of dysbiosis than microbial composition alone. This sensitivity likely arises because BMV production is influenced by bacterial fitness and environmental conditions, reflecting functional output rather than mere genomic presence. Environmental risk factors for IBD, including smoking, diet, hygiene, and antibiotic use, affect the microbiome (268) and may drive functional changes in bacteria, potentially consequently altering their vesiculation process.
We propose that dysbiosis, or functional dysbiosis, results in an altered BMV signaling, which fails to mediate the microbiome's protective and homeostasis-inducing effects. This altered signaling could drive or maintain disrupted homeostasis, exacerbating inflammation and IBD.
Indeed, many protective functions provided by BMVs in healthy hosts appear to be disrupted in IBD.
Patients with IBD exhibit increased intestinal permeability at various sites, including the mucin layer, antimicrobial peptides, and tight junctions (269). Inflammatory cytokines have been shown to downregulate the expression of tight junction-forming proteins (270), further compromising the intestinal barrier in disease conditions. Notably, barrier dysfunction is also observed in inactive CD patients (271). Environmental factors are considered key contributors to this barrier disruption since even spouses of CD patients have shown increased intestinal permeability (272).
IBD Patients also exhibit changes in essential immune mediators of homeostasis. Some studies suggest reduced levels of Tregs in peripheral blood of IBD patients (273) and other studies observe functional changes instead. Tregs seem to differentiate to co-express IL-17, which results in a decrease in their suppressive

capacity. Polymorphisms in the Treg transcription factor FOXP3 are also linked to IBD (274). Additionally, conversion of naive T cells into FOXp3+ cells is impaired under disease conditions (275).
Mutations in IL-10 or its receptor are associated with early onset IBD (276). Monocytes from these patients do not respond with a reduction of proinflammatory cytokines after IL-10 exposure (277).
Other examples include PPAR-γ, which is induced by *F. prausnitzii* OMVs.
UC patients have been found to have reduced PPAR-γ expression (278), and PPAR- γ agonists ameliorate stress-induced intestinal inflammation in mice (279). Accordingly, a lower abundance of *F. prausnitzii* in CD patients is associated with increased risk of disease recurrence (280).
Or IgA, which is induced by AM-OMVs. Patients with IgA deficiency are more predisposed susceptible to IBD (281).

10.3.2 B: The Host must be receptive

On the other hand, the host must adequately process homeostasis inducing OMVs from the microbiome. This requires a variety of sensing molecules to recognize these signals and exert the protective effects of BMVs and preserve homeostasis. IBD is associated with genetic predispositions and these mostly involve disruptions in genes responsible for microbiome interaction. We suggest that these defects impair the bidirectional communication between the host and microbiome.
ATG16L1 has been shown to play an essential role in OMV processing, as it is required for the OMVs protective effect in experimental colitis (91, 92). Again, polymorphisms in the *ATG16L1* gene are associated with CD (282).
Dendritic cells from CD patients homozygous for the ATG16L1 T300A risk variant exhibit defects in promoting protective Foxp3+ Treg development and IL-10 expression after stimulation with *B. fragilis*-OMVs. In mice carrying this variant, the protective effects of *B. fragilis* OMVs were absent, with additional involvement of NOD2 (91).
Being of bacterial origin, BMVs induce the expression of different cytokines. While they induce the regulatory IL-10, other proinflammatory cytokines also seem to be part of the physiological response and important for balancing protection and tolerance.

Mice deficient in MyD88 lack essential TLR-mediated immune responses and experience more severe colitis with increased intestinal damage and significantly higher morbidity and mortality, despite showing reduced levels of pro-inflammatory cytokines (283). This highlights that balanced immune responses, including pro-inflammatory signals, are critical for controlled interaction with the microbiome. This is defective in IBD patients.
For instance, circulating PBMCs from IBD patients treated with *Bacteroides thetaiotaomicron* OMVs showed normal IL-6 responses but significantly reduced IL-10 secretion (100). This imbalance suggests impaired regulatory responses in IBD, as IL-10 typically counteracts IL-6 to maintain immune equilibrium (284). The study also showed that dendritic cells of IBD patients are less responsive to OMV treatment and regulatory Cd103+ DCs counts are reduced.
NOD2 is important for sensing OMVs to initiate diverse downstream effects. NOD2 mutations are associated with IBD, particularly CD (285). In CD patients with NOD2 mutations Paneth cells are defective in their α-defensin production (286). Additionally, NOD2 mutations impair adequate immune processing and cytokine responses. Dendritic cell maturation, IL-10 expression and other cytokine responses are disrupted. These mutations also correlate with a diminished presence of Foxp3+ regulatory T cells in the intestinal mucosa (287-289).

Bacterial sensing and processing is highly relevant for initiating an appropriate immune response. But the mechanisms BMVs utilize to induce tight junction proteins expression are still unexplored. Whether bacterial sensing molecules are required for these barrier-strengthening effects is unknown. However, it has been shown that epithelial cells use NOD1 to sense BMVs (78). NOD1-deficient mice in turn exhibit an impaired colon barrier function and reductions in cadherin and

REG3γ expression (290). However, the causal connection between these observations is only speculation.

10.4 Therapeutic Potential of Bacterial Membrane Vesicles in Inflammatory Bowel Disease

The pathogenesis of Inflammatory bowel diseases is far from solved and poorly understood. The resulting inflammation damages tissue and burdens patient's quality of life. Current treatment strategies rely on immunosuppressants. This approach targets symptoms rather than the root causes of these conditions.

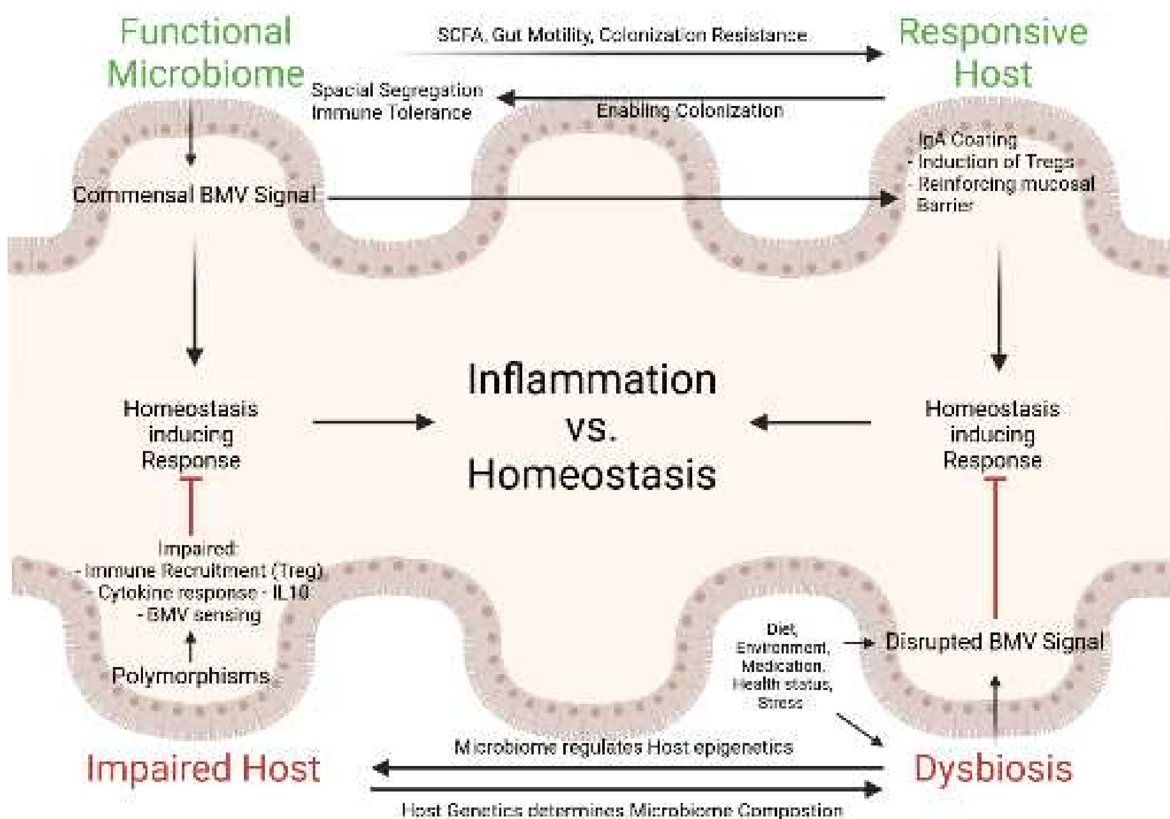


***Figure 8**: The role of Bacterial Membrane Vesicles in Inflammatory Bowel Disease*

The microbiome sends BMVs to establish its colonization by expecting an appropriate homeostasis-inducing response from its host. This relationship can be disrupted on both sides. And when it is, the respective counterpart becomes impaired further.

Further research into the mechanisms driving such diseases is essential to develop more targeted and effective therapies. In IBD leveraging the microbiome for new treatment options is an everlasting goal. Many different approaches have

already been tested.
Probiotics are living microorganisms that can provide benefits for the host. In a meta-analysis, *Escherichia coli Nissle 1917* was shown to be equivalently effective as state-of-the-art mesalacin in maintaining remission in UC patients. In fecal microbiota transplantation (FMT), stool containing a healthy donor's gut bacteria is transferred into a recipient to restore the balance of the gut microbiome. Initial clinical studies of FMT produced inconsistent results, partly due to unstandardized patient selection and co-treatment with immunosuppressants. But still, FMT demonstrated promising remission rates in active UC as well as enhanced maintenance of remission (291-293). Further investigations have identified reasons for the variability in treatment outcomes. Unsurprisingly, the composition of the donor microbiome plays a critical role in the success of the treatment. The abundance of *Akkermansia muciniphila* in the donor microbiome has been correlated with a positive treatment response (241). Unsurprisingly, when donor the donor's microbiome is similar to the recipient's existing flora, chances increase even further (294).
The clinical effects of FMT and probiotics in human IBD patients provide a proof of concept that the microbiome plays an accountable role in IBD's multifactorial pathophysiology (109).
However, both approaches require bacteria to settle in a preexisting environment, which is shaped by individual variables like host genetics, behavior and environment. These conditions may not always be favorable for the transplanted bacteria. As introduced above, changes in microbial presence do not reflect their functionality. Unlike live bacteria, bacterial membrane vesicles are postbiotics released by bacteria, which are less dependent on the preexisting host environment.
Studies with probiotics illustrate the difference. Mice on normal diets showed a greater increase in tight junction protein expression in response to live AM compared to its OMVs. In contrast, mice on a high-fat diet with existing intestinal pathology and low-grade inflammation showed a better response to the OMVs (112).
The individual in vivo situation in patients might not be favorable for certain mechanisms of action but OMVs provide a variety of targets. Another study

illustrates this by showing that OMVs even directly change the microbial composition. This phenomenon was previously only attributed to their anti-inflammatory effect. But indeed, AM-OMVs fuse with bacterial cells to increase their proliferation rate. They even preferentially target commensal bacteria of the natural established flora instead of facultative pathogens. This restores microbial richness and diversity in DSS-colitis (113).

These findings underscore the potential of AM-OMVs as a promising candidate for therapeutic applications for human IBD.

In mice, oral treatment with OMVs has shown promise as a safe and effective therapeutic approach in various conditions, including colon cancer. Although human studies are still lacking, multiple companies are exploring the potential of OMVs for the use as vaccines and cancer immunotherapies (295). OMVs are modified to be used to carry and deliver antigens of interest. Current clinical studies are in Phase I-II trials but there is no robust published data on their safety and efficacy yet. Nevertheless, unmodified OMVs are naturally occurring products that are present in healthy individuals.

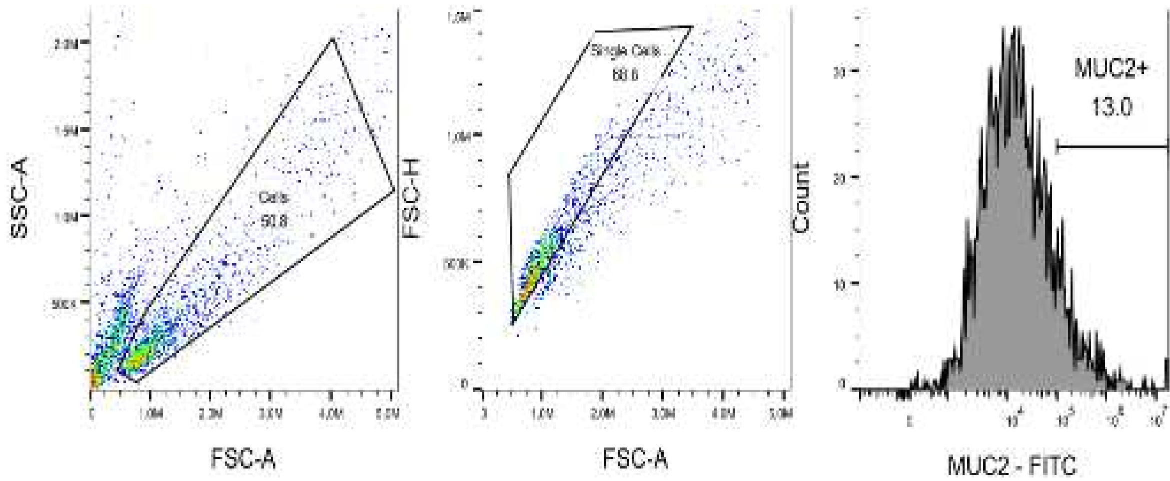
Bacterial membrane vesicles also hold significant potential for individualizing treatment strategies for IBDs, which are multifactorial and vary greatly between individuals. BMVs of different species provide many different routes of sensing and biological effects. With increasing knowledge about these mechanisms, molecules and signaling pathways involved, the BMV therapy could be tailored to the genetics and individual defects of the patient. The identification of bioactive molecules responsible for the BMVs effect is crucial. For example, polysaccharide A (PSA) has been identified as a key bioactive molecule in *Bacteroides fragilis* OMVs (see 7.4.1.3). Similarly, Amuc_1100, a protein carried by *Akkermansia muciniphila* OMVs, has been found to replicate many of the beneficial effects of AM (296). By understanding which molecular pathways are employed by the OMVs along with the potential aberration in microbial sensing genes in patients, we may be able to predict the response to BMV therapy. This would allow the personalized selection of the most appropriate BMV or BMV combination. While this concept is highly theoretical, it opens up possibilities of improving the likelihood of therapeutic success of microbiome-based therapies in IBD.

10.4.1 Challenges

The biology of bacterial membrane vesicles is diverse, complex and influenced by the entirety of their environment. For this reason, the bacteria used to gather BMVs for experiments or potential clinical applications must be cultivated under strictly controlled and comparable conditions. However, standardized protocols are not yet well-established. AM is a good example since it was only discovered in 2004 and culture practices are still being refined for their respective purpose. Using varying cell culture protocols could significantly impact the composition of produced OMVs. Since these compositional differences might influence their therapeutic potential, quality control is essential. The identification of biomarkers can be used to characterize BMVs and identify subsets. Interestingly, it is not known whether BMVs produced under laboratory conditions with specific growth media are identical in composition to those naturally produced by the same species in the intestine. This is why laboratory-produced BMVs and respective experiments have to be carefully evaluated.

The model proposed in this thesis regarding the role of BMVs in the pathophysiology of IBD is currently hypothetical. To validate this model, further research is needed into both the behavior of BMVs in IBD and the general biology of membrane vesicles. The BMV composition is still poorly characterized in IBD. For instance, established environmental risk factors for IBD like smoking and diet should be investigated for their potential to change the BMV signal.

11 Appendix



Appendix 1: *Gating Strategy in Flow Cytometry*

Debris and dead cells were excluded in a SSC-FSC plot.
Doublets were excluded in a FSC-height-FSC-anterior plot.
The resulting population was analyzed in its MUC2 expression. The boarder to classify MUC2 positive cells is arbitrary and excludes outliers off the charts.

Gene	GenBank accession	Orientation	Sequence (5' to 3')
MUC2	NM_002457	F	ACTCTCCACACCCAGCATCATC
		R	GTGTCTCCGTATGTGCCGTTGT
MUC1	NM_002456	F	CCTACCATCCTATGAGCGAGTAC
		R	GCTGGGTTTGTGTAAGAGAGGC
MUC5AC	NM_017511	F	CCACTGGTTCTATGGCAACACC
		R	GCCGAAGTCCAGGCTGTGCG
KLF4	NM_004235	F	CATCTCAAGGCACACCTGCGAA
		R	TCGGTCGCATTTTTGGCACTGG
AGR2	NM_006408	F	GGTGACCAACTCATCTGGACTC
		R	TGACTGTGTGGGCACTCATCCA

Appendix 2: *Primer Sequences for qPCR*

Day 1

Sample	Cell Count	MFI (median)	MFI (mean)	MFI (geometric mean)
Control 1.fcs	3949	6226	19830	6655
Control 2.fcs	3865	6264	17931	6699
Control 3.fcs	3909	5498	18004	6678
DAPT 1.fcs	2996	5274	24808	5880
DAPT 2.fcs	3514	5242	17508	5799
DAPT 3.fcs	3351	5811	18937	5822
OMV 1.fcs	3213	5566	22748	6136
OMV 2.fcs	3730	5824	20415	6407
OMV 3.fcs	3456	5732	18890	6055

Day 4

Sample	Cell Count	MFI (median)	MFI (mean)	MFI (geometric mean)
Control 1.fcs	3526	4442	29137	5087
Control 2.fcs	3473	4339	20171	4782
Control 3.fcs	3882	3920	17977	4434
DAPT 1.fcs	2425	5434	61647	7112
DAPT 2.fcs	3237	4656	52719	6091
DAPT 3.fcs	3198	4823	49674	6349
OMV 1.fcs	3120	4697	38164	5467
OMV 2.fcs	3364	4547	21501	5155
OMV 3.fcs	3513	4141	14236	4696

Day 8

Sample	Cell Count	MFI (median)	MFI (mean)	MFI (geometric mean)
Control 1.fcs	2466	6619	47013	7620
Control 2.fcs	2176	7282	42115	8281
Control 3.fcs	1936	7104	33442	7744
DAPT 1.fcs	2339	6058	70795	7887
DAPT 2.fcs	1896	5842	100919	7906
DAPT 3.fcs	1817	6021	123933	8405
OMV 1.fcs	2824	6264	25656	6978
OMV 2.fcs	2272	6639	35473	7352
OMV 3.fcs	2654	6245	23497	7045

***Appendix 3**: Raw MFI Data*

Raw data from the timeline experiment investigating MUC2 expression is shown to visualize sufficient cell counts and differences between the MFI approaches.

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