Goblet cell intrinsic colonic defense

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என் அன்புக்குரிய குடும்பத்திற்கு

Abstract

The intestine is constantly exposed to billions of commensal microbes as well as pathogens and opportunistic organisms. Protection of the epithelium depends on the mucus barrier, produced by goblet cells (GCs), which separates bacteria from host tissue while maintaining tolerance to the microbiota. Several studies have established the importance of Muc2 and the inner and outer mucus layers in maintaining intestinal homeostasis, but multiple key questions remained unresolved in our understanding of mucus associated protection to bacterial infections. It has been unclear how specialized GC subsets mature after birth, how pathogens selectively undermine these protective cells, and how environmental factors such as diet destabilize mucus integrity to permit disease.

This thesis addresses these gaps by defining the developmental, functional, and pathological dynamics of distinct GC populations. We show that postnatal maturation of sentinel goblet cells (senGCs) is driven by microbial colonization, requiring Duox2 signaling to enable rapid, MAMPs-dependent mucus secretion. In parallel, noncanonical GCs contribute unique proteins to the mucus proteome reinforcing the structural and antimicrobial properties of the inner mucus layer. Building on this, we demonstrate that during *Citrobacter rodentium* infection, the pathogen destabilizes the inner mucus layer and potentially exploits the type III secretion system effector EspF to selectively deplete Spdef-dependent intercrypt GCs (icGCs). Loss of these cells dismantle the intercrypt mucus network, creating niches for bacterial persistence that cannot be compensated by crypt plume mucus alone. Our study further suggests that senGCs are important drivers of crypt-specific secondary defense during infection. They dynamically expand into deep crypts, sustain baseline mucus secretion, and coordinate Th17 responses. Their absence leads to accelerated GC loss, sex-specific susceptibility, and early indications of barrier compromise. Finally, we reveal that short-term exposure to a Western-style diet disrupts jejunal mucus integrity through increased Tgm2-mediated cross-linking of Muc2, preventing proper mucus expansion and enabling ectopic colonization of the small intestine by *C. rodentium.* Together, these findings update our understanding of the intestinal mucus barrier as a dynamic and adaptable system shaped by microbial signals, dietary factors, and contributions from specialized GC subsets. By uncovering how senGCs and icGCs preserve barrier integrity, and how pathogens and diet exploit their vulnerabilities, this work advances mechanistic understanding of how the intestinal GCs and the secreted mucus barriers preserve homeostasis and how their functional ablation creates vulnerabilities during infection.

Keywords: Goblet cells, enteric pathogens, mucosal defence

Populärvetenskaplig sammanfattning

Våra tarmar är hem för biljoner bakterier, varav de flesta är ofarliga eller till och med bra för vår hälsa. För att vi ska kunna leva tillsammans med dessa bakterier är tarmen täckt av ett skyddande slemlager som ser till att bakterierna inte kan nå tarmväggen. Denna slembarriär byggs och upprätthålls av speciella celler, så kallade bägarceller vars främsta uppgift är att producera och frisätta slem. Min forskning har fokuserat på att studera hur slembarriären utvecklas tidigt i livet, hur den påverkas av kosten, och hur sjukdomsalstrande bakterier bryter sig igenom slembarriären för att infektera tarmen.

Våra resultat visar att i nyfödda råttor använder tarmen ett tillfälligt ”spolningssystem” som sköljer bort bakterier till dess att slembarriären har utvecklats. När råttorna växer upp och utsätts för en ökad mängd bakterier aktiveras en speciell typ av bägarceller så kallade sentinel-bägarceller. Dessa celler fungerar som vaktposter som står vid öppningarna till tarmslemhinnans kryptor och känner av hur mycket bakterier som finns i omgivningen. Om mängden bakterier blir för hög stimulerar de frisättning av slem som flyttar bakterierna bort från tarmcellerna. Tillsammans visar dessa resultat att tarmen har olika typer av skyddssystem vid olika tidpunkter i livet, och att kroppen anpassar sig till förändringar som sker i tarmens lokala miljö. När vi studerade hur bakterien *Citrobacter rodentium* (en musmodell för *E. coli* infektion i människa), koloniserar tarmen upptäckte vi att skyddet från slemlagret varierade över tarmytan. En undergrupp av bägarceller så kallade interkrypt-bägarceller producerar ett mer genomsläppligt slemlager som lägger sig mellan kryptorna, medan kryptorna är täckta av ett mer kompakt slemlager. För att kolonisera tarmen har *C. rodentium* utvecklat ett smart trick: den använder proteinet EspF för att förstöra interkrypt-bägarcellerna och bryter ner det tunnare slemlagret mellan kryptorna och kan på så sätt komma ner till tarmytan och stanna kvar där. Dessa resultat visar hur sjukdomsalstrande bakterier har hittat slembarriärens svaga punkter och utnyttjar dessa för att infektera tarmen. Våra resultat visar även att sentinel-bägarcellerna inte bara är passiva vakter utan de skyddar även kryptorna mot infektion. När *C. Rodentium* etablerar sig i tarmen flyttar sentinel-bägarcellerna ner i kryptorna där de producerar mer slem och hjälper andra bägarceller att överleva. Utan sentinel-bägarcellerna kollapsar slembarriären snabbare och bakterierna har lättare att invadera vävnaden. Vi fann även att i möss som saknar sentinel-bägarceller var honmöss mer infektionskänsliga än hanmöss, vilket visar på könsspecifika skillnader i tarmens skyddssystem. Denna upptäckt öppnar för frågan om liknande skillnader finns hos människa och om kön påverkar hur vi hanterar tarminfektioner.

Vår forskning visade även att kosten påverkar slembarriären. En kort period med västerländsk diet (hög mängd fett och brist på kostfibrer) försvagade slemlagret i tunntarmen och skapade plats för bakterier som normalt sett inte kan kolonisera tunntarmen. Dessa resultat visar att det inte bara är infektioner som påverkar tarmens försvarssystem utan även livsstil. Sammanfattningsvis visar den här avhandlingen att tarmens slemlager är ett dynamiskt försvarssystem bestående av flera olika komponenter som tillsammans skyddar oss mot både våra egna tarmbakterier och sjukdomsalstrande bakterier, och att både kön och livsstil påverkar den här skyddsbarriären.

பொதுமக்கள் அறிவியல் சுருக்கம்

மனிதக்குடல் கோடிக்கணக்கான நுண்ணுயிரிகளை (மிக்ரோப்கள்) தங்கவைக்கிறது. அவற்றில் பெரும்பாலானவை நன்மை பயப்பவையாக இருந்தாலும், சில தீங்கு விளைவிக்கக்கூடியவை. இந்த நுண்ணுயிரிகளை கட்டுப்படுத்த குடல் ஒரு பாதுகாப்பு கவசத்தைப் போலச் செயல்படும் பிசின் (mucus) அடுக்கால் மூடப்பட்டுள்ளது. இந்த பிசி அடுக்கை "கோப்லெட் செல்கள்" (goblet cells) எனப்படும் சிறப்பு செல்கள் தொடர்ந்து உற்பத்தி செய்கின்றன. என் ஆய்வுகள் இந்த பிசி அடுக்கு எவ்வாறு உருவாகிறது, உணவு மற்றும் தொற்றுகள் அதை எவ்வாறு பாதிக்கின்றன, மற்றும் தீங்கு விளைவிக்கும் பாக்டீரியாக்கள் அதை எவ்வாறு உடைக்கின்றன என்பதைக் கவனித்தன.

பிறந்த குழந்தைகளில், குடல் முதலில் தற்காலிக "கழுவும்" முறையைப் பயன்படுத்தி நுண்ணுயிரிகளை வெளியேற்றுகிறது எனக்கண்டறிந்தோம். பின்னர், வளர்ச்சியுடன், "சென்டினல் கோப்லெட் செல்கள்" (sentinel GCs) எனப்படும் சிறப்பு செல்கள் செயல்படத் தொடங்குகின்றன. இவை குடல் சுவரில் உள்ள சிறிய குழிகளின் (crypts) வாயிலில் காவலர்களைப் போல நின்று, பாக்டீரியாவை உணர்ந்து உடனடியாக பிசி வெளியேற்றி அவற்றைத் தடுக்கின்றன.

நாங்கள் *Citrobacter rodentium* எனப்படும் பாக்டீரியாவால் ஏற்படும் குடல் தொற்றை ஆய்வு செய்தபோது, பிசி அடுக்கு ஒரே மாதிரியானது அல்ல என்பதை கண்டறிந்தோம். "இன்டர்கிரிப்ட் கோப்லெட் செல்கள்" (intercrypt GCs) எனப்படும் மற்றொரு சிறப்பு குழு, குடல் குழிகளுக்கிடையே நுண்ணிய பிசி வலை ஒன்றை உருவாக்குகிறது. இது பாக்டீரியாவை குடல் சுவரை அடையாமல் தடுக்கிறது. ஆனால் அந்த பாக்டீரியா EspF எனப்படும் ஒரு சத்தைப் பயன்படுத்தி இந்த செல்களை அழித்து, அந்த வலைப்பாதையை உடைத்து, குடல் மேற்பரப்பில் நிலைத்திருக்கிறது.

மேலும், சென்டினல் கோப்லெட் செல்கள் வெறும் காவலர்கள் அல்ல என்பதை நாங்கள் காட்டினோம். தொற்றின் போது, அவை ஆழமான குழிகளுக்குள் நகர்ந்து, அதிக பிசி உற்பத்தி செய்து, பிற கோப்லெட் செல்களை உயிர்வாழ உதவுகின்றன. இவை இல்லையெனில், பாதுகாப்பு விரைவாக சிதைந்து, பாக்டீரியாவுக்கு எளிதில் நுழைய

வாய்ப்பு கிடைக்கிறது. சுவாரஸ்யமாக, பெண் எலிகள் இந்த செல்கள் இல்லாதபோது அதிக பாதிப்புக்குள்ளானது, இது பாலின அடிப்படையிலான வேறுபாடுகளை காட்டுகிறது. இறுதியாக, உணவும் இந்த பாதுகாப்பை பாதிக்கக்கூடும் என்பதை நாங்கள் கண்டறிந்தோம். குறுகிய காலம் மேற்கத்திய உணவுமுறையைப் பின்பற்றியதில், சிறுகுடலில் பிசி அடுக்கு பாதிக்கப்பட்டது. இதனால், சாதாரணமாக கிருமிகள் குடியேற முடியாத, இயல்பாகத் தூய்மையான பகுதிகளிலும் பாக்டீரியாக்கள் தங்குவதற்கு புதிய வாய்ப்புகள் உருவானது எனக்கண்டறிந்தோம்.

மொத்தத்தில், இந்த ஆய்வுகள் குடலின் பிசி அடுக்கு ஒரு நிலையான சுவர் அல்ல, மாறாக பல அடுக்குகளைக் கொண்ட, தன்னிச்சையாக மாறும் பாதுகாப்பு அமைப்பு என்பதை வெளிப்படுத்துகின்றன. வெவ்வேறு கோப்லெட் செல்கள் தனித்தனி பங்குகளை வகிக்கின்றன, நோய்க்கிருமிகள் பலவீனங்களைப் பயன்படுத்துகின்றன, மேலும் உணவு இந்த சமநிலையை குலைக்கக்கூடும். இந்த செயல்முறைகளைப் புரிந்துகொள்வது, வாழ்க்கையின் ஆரம்பத்தில், தொற்றுகளின் போது, மற்றும் நவீன உணவுமுறைகளின் தாக்கத்தில் குடல் பாதுகாப்பை வலுப்படுத்த புதிய வழிகளைத் தேட உதவும்.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Åsa Johansson*, Mahadevan Venkita Subramani*, Yilmaz B, Nyström EEL, Layunta E, Arike L, Sommer F, Rosenstiel P, Vereecke L, Mannerås-Holm L, Wullaert A, Pelaseyed T, Johansson MEV, Birchenough GMH. **Neonatal microbiota colonization primes maturation of goblet cell-mediated protection in the pre-weaning colon.** J Exp Med. 2025. *co-first author (equal contribution)

II. Mahadevan Venkita Subramani, Juan S. Bailo, Alexandra Thiran, Aimee Reddy, Karl T Hansson, Lars Vereecke, Elisabeth E.L. Nyström, Jenny Gustafsson, George M.H. Birchenough. **Citrobacter rodentium infection disrupts colonic mucus barrier function via ablation of intercrypt goblet cells**. Manuscript

III. Mahadevan Venkita Subramani, Lars Vereecke, Jenny Gustafsson, Elisabeth E.L. Nyström, George M.H. Birchenough. **Nlrp6-dependent sentinel goblet cell functions dictate defensive responses during bacterial invasion of colonic crypts.** Manuscript

IV. George M.H. Birchenough, Bjoern O. Schroeder, Sinan Sharba, Liisa Arike, Christian V. Recktenwald, Fabiola Puertolas-Balint, Mahadevan Venkita Subramani, Karl T. Hansson, Bahtiyar Yilmaz, Sara K. Linden, Fredrik Backhed & Gunnar C. Hansson. **Muc2-dependent microbial colonization of the jejunal mucus layer is diet sensitive and confers local resistance to enteric pathogen infection.** Cell reports. 2023

Contents

Abbreviations

AGR2 - Anterior Gradient 2
Ang4 - Angiogenin 4
AQP8 - Aquaporin 8
ASC - Apoptosis-Associated Speck-Like Protein Containing a CARD
BCAS1 - Breast Carcinoma Amplified Sequence 1
CCH - Colonic Crypt Hyperplasia
CD - Crohn's Disease
CD4 - Cluster of Differentiation 4
CD8 - Cluster of Differentiation 8
CFU - Colony Forming Unit
CLCA1 - Calcium-Activated Chloride Channel Regulator 1
ConvD - Conventionalized
ConvR - Conventionally Raised
Cts - Cathepsin
DMBT1 - Deleted in Malignant Brain Tumors 1
dpi - Days Post-Infection
DSS - Dextran Sodium Sulfate
Duox2 - Dual Oxidase 2
EHEC - Enterohemorrhagic Escherichia coli
EPEC - Enteropathogenic Escherichia coli
ER - Endoplasmic Reticulum
EspF - Escherichia coli Secreted Protein F
FCGBP - Fc Gamma Binding Protein
FML - Faecal Mucus Layer
FOXA3 - Forkhead Box A3
GC - Goblet Cell
GF - Germ-Free
GFP - Green Fluorescent Protein
GSDMC4 - Gasdermin C4
IBD - Inflammatory Bowel Disease
icGC - Intercrypt Goblet Cell
IECs - Intestinal Epithelial Cells
IFNγ - Interferon Gamma
Igha - Immunoglobulin Heavy Constant Alpha
Igkv733 - Immunoglobulin Kappa Variable 7-33
Igkv828 - Immunoglobulin Kappa Variable 8-28
IL- Interleukin

IML - Inner Mucus Layer
IRE1β - Inositol-Requiring Enzyme 1 Beta
KO - Knockout
LB - Luria-Bertani (broth)
LEE - Locus of Enterocyte Effacement
Lgals3 - Galectin-3
MAMPs - Microbe-Associated Molecular Patterns
Mep1a - Meprin A Subunit Alpha
MKI67 - Marker of Proliferation Ki-67
MLN - Mesenteric Lymph Node
MUC – Mucin gene
Muc2iCre - Mucin 2 Promoter-Driven Inducible Cre Recombinase
MXD1 - MAX Dimerization Protein 1
MyD88 - Myeloid Differentiation Primary Response 88
NLRC4 - NLR Family CARD Domain-Containing Protein 4
NLRP - NLR Family Pyrin Domain-Containing Protein
$Nlrp6^{fl/fl}$ - Floxed Nlrp6 Allele
$Nlrp6^{\Delta GC}$ - Goblet Cell-Specific Nlrp6 Knockout
NOD2 - Nucleotide-Binding Oligomerization Domain-Containing Protein 2
P3CSK4 - Synthetic Triacylated Lipopeptide (TLR2/1 Agonist)
PDIA - Protein Disulfide Isomerase A
PIEZO1 - Piezo-Type Mechanosensitive Ion Channel Component 1
RAB - Ras-Related Protein
REG - Regenerating Islet-Derived Protein
REP15 - Rab15 Effector Protein
RNAseq - RNA Sequencing
Rorγt - RAR-Related Orphan Receptor Gamma t
ROS - Reactive Oxygen Species
senGC - Sentinel Goblet Cell
SI5 - Segment 5 of Small Intestine
SLC26A3 - Solute Carrier Family 26 Member 3
SLFN4 - Schlafen Family Member 4
SPDEF - SAM Pointed Domain Containing ETS Transcription Factor
STXBP1 - Syntaxin Binding Protein 1
SYTL2 - Synaptotagmin-Like Protein 2
T3SS - Type III Secretion System
TA - Transit Amplifying
TGM - Transglutaminase
Th - T Helper

Tir - Translocated Intimin Receptor
TLR - Toll-Like Receptor
TRIF - TIR-Domain-Containing Adapter-Inducing Interferon-β
UC - Ulcerative Colitis
UEA-1 - Ulex Europaeus Agglutinin I
VAMP8 - Vesicle-Associated Membrane Protein 8
WGA - Wheat Germ Agglutinin
WSD - Western-Style Diet
WT - Wild Type
Zg16 - Zymogen Granule Protein 16
ΔescN - EscN Gene Knockout

1 Introduction

1.1 Intestinal mucosal surfaces

The intestinal epithelium represents one of the most demanding environments in the body as it must coexist with billions of commensal microbes as well as opportunistic pathogens that together outnumber human cells. In order to cope with this constant exposure, the intestine has evolved structural and functional adaptations that allow it to maintain balance while supporting the body's survival and growth [1]. The small intestine is specialized for nutrient absorption and the forward movement of digested material, ensuring that essential compounds are taken up efficiently. In contrast, the colon has developed a more complex system that integrates dense microbial communities, their secreted metabolites, and layered mucus structures. These elements work together to process dietary components that escape digestion in the small intestine, recover additional energy, and ultimately prepare waste for excretion. In this way, the intestinal epithelium sustains both metabolic needs and protective functions within a highly dynamic environment [2, 3].

1.1.1 Mucus organization and structural composition

The intestinal mucus barrier is primarily composed of the gel-forming mucin MUC2, which is produced and secreted by goblet cells (GCs) [4]. Of the five known gel-forming mucins secreted by GCs, four (MUC2, MUC5AC, MUC5B, and MUC6) are present in humans, although not all are expressed in the intestine [4]. In addition to secreted mucins, the intestinal epithelium expresses approximately eleven transmembrane mucins that remain anchored to the cell surface and potentially contribute to barrier function, lubrication, and cell signaling [4].

GCs also release several core proteins that support maintenance of mucus structure and function. These include the Fc gamma binding protein (FCGBP) and the calcium-activated chloride channel regulator 1 (CLCA1), both of which help define the mucus network [3, 5, 6]. The mucus layer also contains epithelial cell byproducts, expelled cellular components, serum proteins such as hemoglobin and albumin, and metabolites released by the gut microbiota. These components show the composite biochemical nature of the mucus barrier, and its contribution to the diversity of the mucosal interface [3, 7].

Before secretion, MUC2 undergoes extensive intracellular processing in the endoplasmic reticulum and Golgi apparatus. This process requires specialized

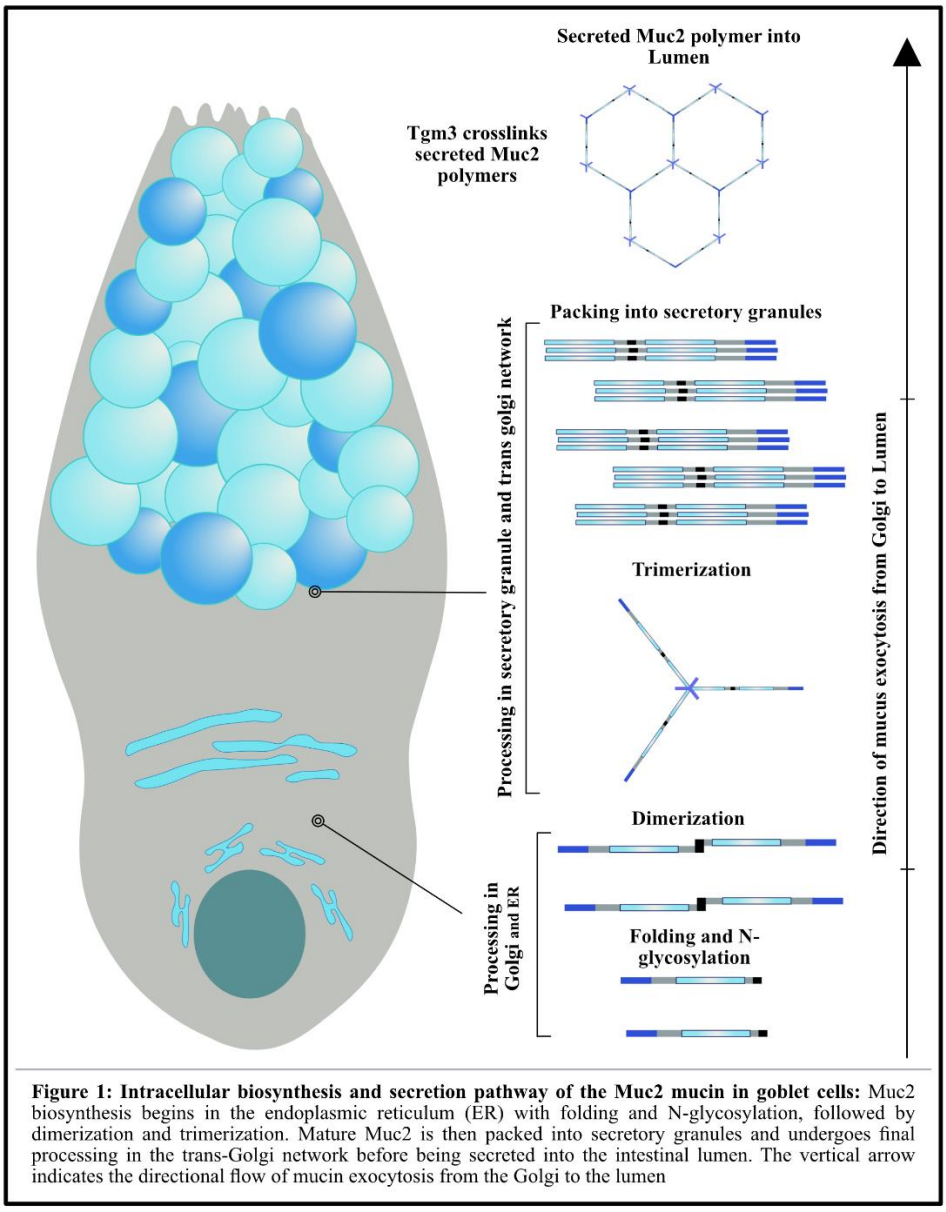

Figure 1: Intracellular biosynthesis and secretion pathway of the Muc2 mucin in goblet cells: Muc2 biosynthesis begins in the endoplasmic reticulum (ER) with folding and N-glycosylation, followed by dimerization and trimerization. Mature Muc2 is then packed into secretory granules and undergoes final processing in the trans-Golgi network before being secreted into the intestinal lumen. The vertical arrow indicates the directional flow of mucin exocytosis from the Golgi to the lumen

chaperones such as IRE1β, AGR2, and several members of the PDIA protein family [8, 9]. Within the Golgi and ER, MUC2 is further modified through glycosylation and folding which are essential for its structural integrity and resistance to proteolysis [10, 11]. The processed mucus is then stored in secretory vesicles under low pH and high calcium conditions in order to aid compact packaging and regulated release [10] (Figure 1).

GCs can release mucus both at a steady baseline rate and in response to external stimuli (discussed separately in later sections). Upon secretion, the mucus expands with the help of bicarbonate ions and forms a dense, layered network. Transglutaminase enzymes stabilize this network by forming covalent bonds between glutamine and lysine residues on MUC2 polymers. TGM3 is the most abundantly expressed transglutaminase in the colon that catalyses this crosslinking reaction primarily on the secreted MUC2 polymers of colonic crypt plume mucus. Lack of TGM3 renders the MUC2 protein increasingly sensitive to proteolytic degradation. Additionally, mice that lacked Tgm3 have been shown to be more susceptible to chemically induced colitis [12]. By contrast, in the small intestine TGM3 is not expressed, although some TGM2 activity has been reported. Meanwhile, proteins like CLCA1 can loosen the mucus in colon through proteolytic cleavage further supporting peristalsis and possibly facilitating ion movement within the mucus layer [5]. This balance between stabilization and controlled breakdown is essential for maintaining an effective and homeostatic intestinal barrier.

1.1.2 Mucus layer in the small intestine

The mucus layer in the small intestine is relatively thin and more permeable which likely supports its primary role in absorbing nutrients from digested food. This looser structure allows nutrients to reach the epithelial surface more easily and can also help in delivering drug molecules for faster uptake [13, 14]. In addition to this, the mucus secretory response in the small intestine can stimulate the release of digestive enzymes and assist in moving digested material toward the colon through peristalsis. In the distal part of the small intestine (ileum), the environment becomes a bit more complicated due to a gradual increase in bacterial presence. Here, GCs and Paneth cells coordinate to release antimicrobial peptides and fluids that act against incoming microbes. These secretions may form concentration gradients along the crypt-villus axis, creating localized zones of protection [15, 16]. This combined activity helps maintain a balance between allowing nutrient absorption and preventing microbial intrusion, ensuring that the small intestine remains both functional and protected as it transitions toward the colon (Figure 2).

1.1.3 Mucus layer in the colon

The colonic epithelium serves a distinct and specialized role, shaped by a densely populated and dynamic microenvironment. This region faces constant mechanical stress from the movement of bulky fecal matter along with exposure to luminal threats and potential pathogens that may breach the mucus barrier [17, 18]. To manage these challenges, the colonic epithelium relies on a physical protective layer of mucus that

separates microbes from host tissue. Unlike the small intestine, the colon features a two-layered mucus system: a dense inner layer that is typically free of bacteria, and a loosely organized outer layer that houses the gut microbiota [18, 19] (Figure 2). In the proximal colon, the mucus surrounds fecal matter and can contain bacteria near the epithelial surface, but secretions from colonic crypts usually help maintain a mostly sterile interface [3]. While many features of the colon are shared between mice and humans, the proximal colon shows structural differences across species [20].

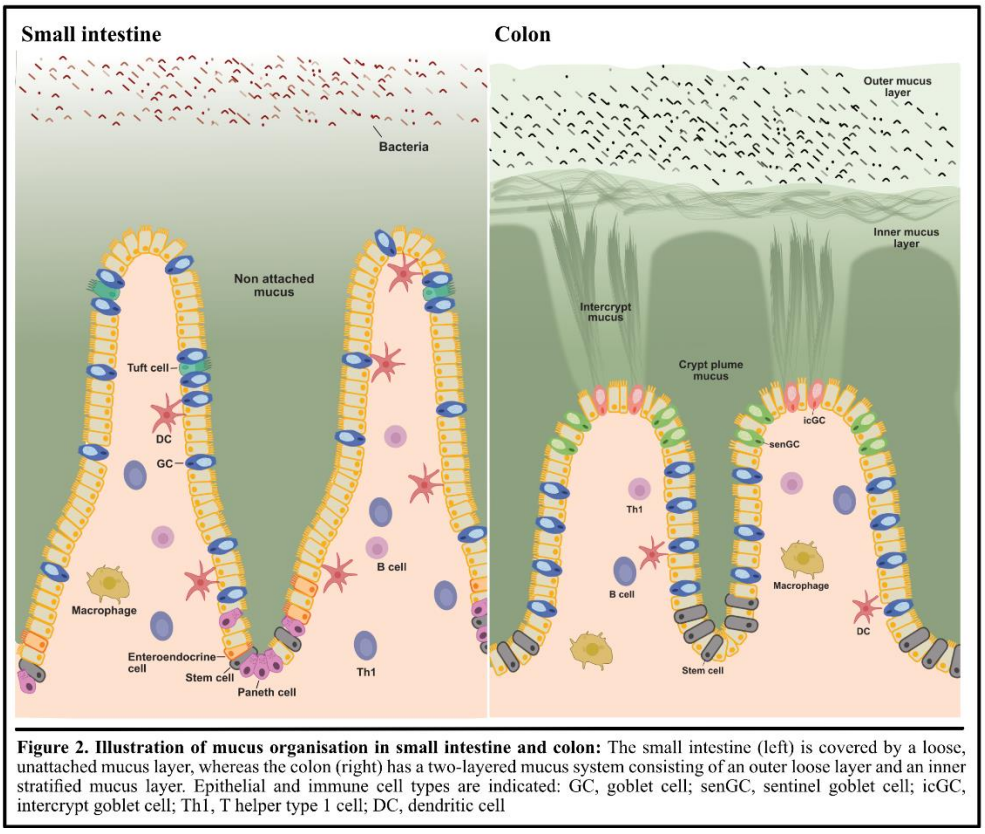

Figure 2. Illustration of mucus organisation in small intestine and colon: The small intestine (left) is covered by a loose, unattached mucus layer, whereas the colon (right) has a two-layered mucus system consisting of an outer loose layer and an inner stratified mucus layer. Epithelial and immune cell types are indicated: GC, goblet cell; senGC, sentinel goblet cell; icGC, intercrypt goblet cell; Th1, T helper type 1 cell; DC, dendritic cell

The inner mucus layer is organized through distinct substructures, including crypt plume mucus and intercrypt mucus (described later in detail), which together form a stable and interconnected barrier that limits bacterial access [21]. Interestingly, some bacteria are known to feed on the carbohydrate side chains of mucin polymers, forming localized symbiotic relationships with the host epithelium [22]. This interaction highlights the balance between microbial presence and epithelial protection in the colon.

1.2 Colonic goblet cell heterogeneity

Early descriptions of intestinal goblet cells treated them as a uniform epithelial population, their function largely reduced to the production and secretion of Muc2, the gel-forming mucin that constitutes the intestinal mucus barrier [1, 23, 24]. With the development of single-cell transcriptomic technologies and the ability to profile gene expression at high resolution, this simplistic view has been revised. It is now clear that goblet cells are not a monolithic group but instead display substantial heterogeneity, with distinct transcriptional programs and region-specific functions. Recent studies have demonstrated that goblet cells can be divided into subpopulations with unique gene expression profiles and specialized roles [21]. For example, one investigation highlighted the induction of a particular goblet cell subset in patients with ulcerative colitis, suggesting that disease states can selectively expand or activate certain GC types [25]. Building on this, subsequent single-cell analyses provided a more comprehensive classification, defining goblet cells as canonical, non-canonical, or proliferative, thereby firmly establishing their heterogeneity [21].

Canonical goblet cells are characterized by expression of classical GC genes such as *Muc2, Fcgbp, Clca1,* and *Atoh1*. These genes are associated with lineage commitment, mucus production, and the maintenance of epithelial barrier function [5, 21, 26, 27]. Non-canonical goblet cells, in contrast, express genes more typically linked to enterocytes and defense responses, including *Muc17, Gsdmc4, Slc26a3,* and *Dmbt1 [21]*. Interestingly, *Hes1*, a transcription factor known to negatively regulate goblet cell differentiation is enriched in this non-canonical group which shows their divergent developmental trajectory [28-30]. Proliferative goblet cells marked by *mKi67* expression are generally located at the crypt base and represent a pool of actively cycling cells. While the precise contributions of each subset to barrier maintenance remain under investigation, canonical goblet cells are widely considered the primary source of the colonic mucus barrier [21]. Non-canonical goblet cells contribute to additional functions such as the secretion of digestive enzymes (e.g., sucrase-isomaltase) and antimicrobial peptides linking them to both digestive and bactericidal roles [21, 24].

The differentiation of goblet cells has been elucidated using animal models and intestinal organoids [23, 31]. These studies show that goblet cells arise from stem cells at the crypt base and migrate upwards, with a turnover time of approximately 5–7 days [32]. Differentiation is tightly regulated by signaling pathways such as Notch and Wnt that act as negative regulators, and inhibition of these pathways promotes goblet cell maturation [33, 34]. The process occurs in two stages. First, lineage commitment is transcriptionally driven by *Atoh1 [27-29]*. Subsequently, further

differentiation and acquisition of functional identity are directed by factors such as *Gfi1, Spdef,* and *Spink4 [27]*. Additional regulators, including *Bcas1, Rep15,* and *Foxa3*, have been implicated in fine-tuning goblet cell lineage progression [21, 24].

Recent pseudo time analyses have provided further insight into goblet cell maturation. Both canonical and non-canonical goblet cells appear to originate from *mKi67+* proliferative precursors at the crypt base, diverging along distinct developmental trajectories [21, 24]. Terminally differentiated cells in both lineages express *Mxd1*, a marker enriched in mature cells, as well as *Slfn4* and *Aqp8*, genes associated with upper-crypt goblet cells. This complex developmental landscape highlights the plasticity of goblet cells enabling them to adapt rapidly to environmental changes such as dietary shifts or microbial challenges. The recognition of this heterogeneity has shifted the field from a simplistic model to a nuanced appreciation of goblet cells as a dynamic and adaptable epithelial population [21].

1.2.1 The intercrypt goblet cells

Intercrypt goblet cells (icGCs) are positioned between adjacent crypts at the epithelial surface. These cells are *Mxd1+Slfn4+*, show negative regulation of differentiation, and express genes associated with bacterial sensing and stress responses likely demanded by their exposed location. Histologically, icGCs can be distinguished by Ulex europaeus agglutinin I (UEA-1) staining, whereas wheat germ agglutinin (WGA) typically marks crypt-resident goblet cells. Functionally, icGCs differ from crypt goblet cells in their secretion dynamics. Rather than storing large quantities of Muc2 in a theca before release, icGCs secrete mucus rapidly, a feature supported by their lower expression of protein-folding genes such as *Agr2* and the secretion regulator *Stxbp1*. Although icGCs express many canonical GC genes (*Muc2, Clca1, Fcgbp*), their function is critically dependent on the transcription factor *Spdef [21, 29]*.

The mucus secreted by icGCs fills the spaces between crypt plumes and is structurally distinct. Produced at a faster rate, it is permeable to small molecules, unlike the dense crypt mucus that excludes both bacteria and solutes. This difference further highlights the functional specialization of icGCs (Figure 3). Loss of *Spdef* disrupts icGCs and their mucus, leading to both spontaneous, age-dependent inflammation and increased susceptibility to DSS-induced colitis. Interestingly, Spdef deficiency impairs icGC function without altering crypt GC features such as theca size or ER morphology. Moreover, although intercrypt mucus produced by *Spdef*$^{-/-}$ mice is less mature, their turnover rates remain comparable to crypt GCs. Importantly, icGCs are reduced in

ulcerative colitis patients again cementing their protective role in maintaining epithelial homeostasis [21].

The recognition of icGCs as a distinct subset has broadened our understanding of how mucus is organized in the colon. Rather than a uniform sheet, the mucus barrier is a patchwork of overlapping structures, with crypt plumes providing dense, impenetrable protection and intercrypt mucus offering a more permeable but rapidly replenished layer. This arrangement may allow the intestine to balance two competing needs such as keeping bacteria at bay while still permitting the diffusion of nutrients and signaling molecules.

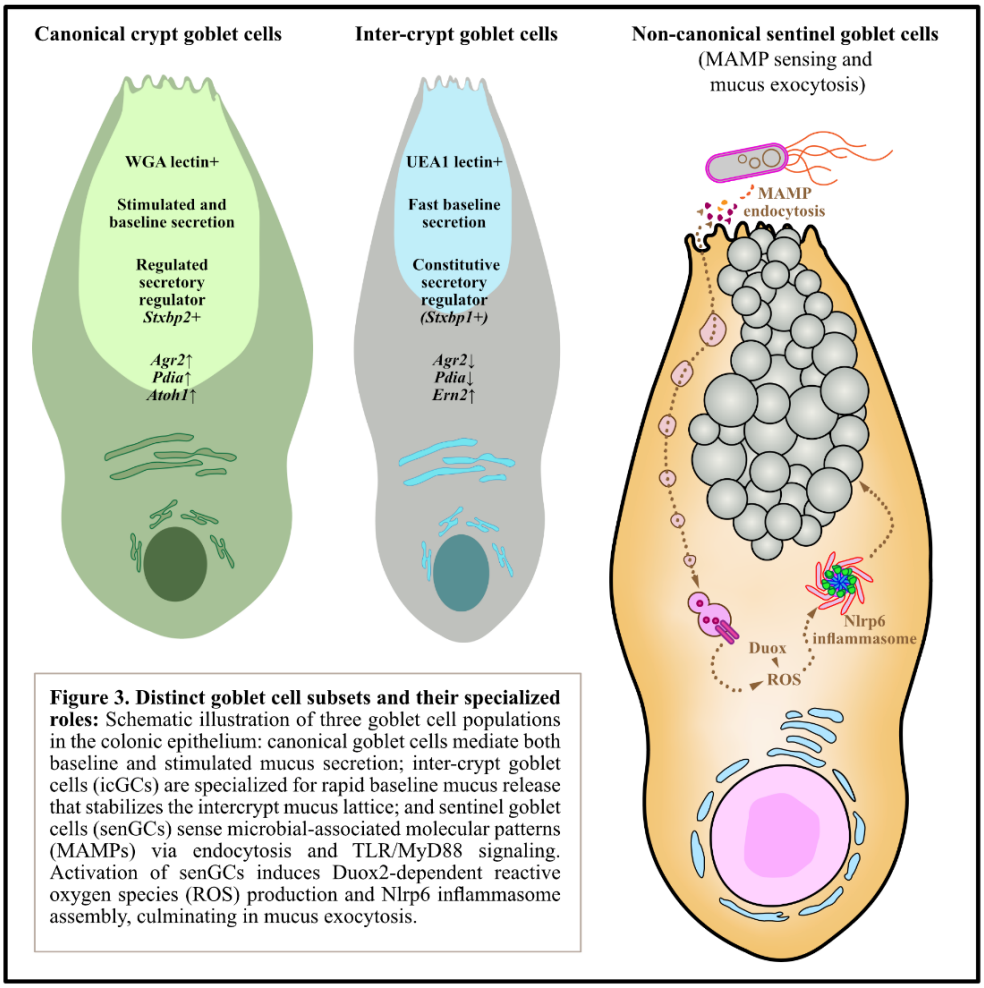

Figure 3. Distinct goblet cell subsets and their specialized roles: Schematic illustration of three goblet cell populations in the colonic epithelium: canonical goblet cells mediate both baseline and stimulated mucus secretion; inter-crypt goblet cells (icGCs) are specialized for rapid baseline mucus release that stabilizes the intercrypt mucus lattice; and sentinel goblet cells (senGCs) sense microbial-associated molecular patterns (MAMPs) via endocytosis and TLR/MyD88 signaling. Activation of senGCs induces Duox2-dependent reactive oxygen species (ROS) production and Nlrp6 inflammasome assembly, culminating in mucus exocytosis.

1.2.2 The sentinel goblet cells

A distinct subset of non-canonical goblet cells located at crypt entrances are termed sentinel goblet cells (senGCs). These cells are enriched for *Nlrp6,* along with other non-canonical genes such as *Dmbt1, Tgm3,* and *Gsdmc4* [21]. The senGCs can endocytose bacterial microbe-associated molecular patterns (MAMPs) at concentrations higher than those normally present in the mucus layer. They then respond to these ligands in a TLR-dependent manner, activating MyD88-mediated Nox/Duox ROS production and assemble the *Nlrp6* inflammasome. Our own work (Paper I in this thesis) demonstrated that *Duox2*, but not *Nox1*, is essential for senGC activation. Inflammasome signaling triggers calcium-dependent compound exocytosis of Muc2. Activated senGCs then transmit signals via gap junctions to neighboring goblet cells coordinating a broad mucus release that expels bacterial products in a time dependent manner. Following this response, senGCs are shed from the epithelial surface [35] (Figure 3).

The sentinel goblet cell response represents a striking example of how epithelial cells can act as first responders to microbial threats. By detecting bacterial products and initiating a coordinated mucus release, senGCs function can potentially act as gatekeepers of the crypts, preventing microbes from penetrating deeper into the tissue. Their eventual ejection from the epithelium after activation could suggest a sacrificial role where individual cells are lost to preserve the integrity of the barrier. While in vivo roles of senGCs are still being defined (and demonstrated in this thesis as part of Paper III), reduced senGC frequency and impaired MAMP-induced secretion have been observed in ulcerative colitis, highlighting their importance as guardians of crypt entrances [6].

1.2.3 Mucus production and secretion machinery

The position of goblet cells along the crypt axis influences the dynamics of mucus biosynthesis. Surface goblet cells secrete mucus at a faster rate compared to crypt-resident cells [36]. Producing Muc2 is a demanding task, as it is a very large glycoprotein that requires complex synthesis, extensive modification, and careful packaging [10, 11, 37]. Mucins are synthesized in the ER and Golgi, where they undergo glycosylation, dimerization, and trimerization before being packed into apical granules [9].

Specialized trafficking proteins, including SNAREs and Rab family members (SYTL2, RAB27A, RAB27B), regulate the organization and transport of mucin vesicles [21]. Goblet cell-specific proteins such as the chaperone AGR2 and the ER

stress sensor IRE1β (ERN2) further support this process, and their absence can impair mucus production and promote inflammation [38-40]. Mucus release is mediated by proteins such as Vamp8 (a SNARE) and Stxbp1, which regulate baseline secretion [41, 42]. Beyond constitutive release, secretion can also be stimulated by multiple pathways. The mechanosensitive ion channel PIEZO1 detects luminal pressure and triggers mucus release, while cytokines, cholinergic agents, and histamine can also induce secretion in the colon [43-45]. Prostaglandins has been shown to stimulate mucus release in the small intestine [46]. As mentioned earlier, senGCs respond to microbial cues with compound exocytosis, and more recently nociceptor-derived neuropeptides have also been shown to activate goblet cell secretion in the colon [47]. These pathways based on previous studies highlight the highly coordinated machinery that enables goblet cells to sustain mucus production and adapt their secretory responses to both physiological and environmental challenges.

1.3 The role of mucus in intestinal homeostasis, disease and its implications

Goblet cells and the mucus they produce form the first line of defense in the intestine by physically separating bacteria from the epithelial surface. This barrier, however, is not static. The mucus layer is constantly renewed and adjusted, allowing the intestine to recover from short-term disturbances caused by diet, infection, or other environmental factors [35, 48, 49]. When this dynamic protection is only briefly disturbed by acute inflammatory triggers, the system usually restores balance in a timely manner. But when the disruption is prolonged or repeated, the breakdown of mucus homeostasis can become an early driver of chronic inflammatory conditions [50-53]. Evidence from mouse models of colitis has shown that weakening of the mucus barrier permits bacteria to cross into the epithelium, which in turn initiates inflammatory responses and disease onset [50, 52].

1.3.1 Colitis

Chronic inflammatory bowel diseases (IBD) are broadly divided into two major forms: ulcerative colitis (UC) and Crohn's disease (CD). Both have been studied extensively for decades helping us understand that the driving causes are multifactorial. Pathogens, diet, environmental triggers, immune dysregulation, and genetic predisposition can all contribute often in combination to disease development. Across these diverse etiologies, one recurring theme is the link between disease and a compromised mucus barrier or dysfunctional goblet cells [54].

For many years, researchers debated whether inflammation of the intestine allows bacteria to reach the epithelial surface, or whether bacterial penetration of the mucus barrier is the initiating event that drives inflammation. Recent work in mouse models of colitis has provided clarity, showing that bacterial entry through the mucus occurs upstream of tissue inflammation [50]. This suggests that barrier failure is not simply a consequence of inflammation but can be a primary trigger.

Clinical studies support this view. In one study of 28 patients with active UC, the mucus layer was found to be thinner and more permeable to bacteria [52]. Another investigation in UC patients revealed alterations in core mucus proteins, particularly MUC2 and FCGBP, which are central to mucus structure and stability [6]. Genome wide analyses of Crohn's disease samples have also pointed to altered goblet cell function, with involvement of bacterial sensing pathways, endoplasmic reticulum (ER) stress responses, and autophagy in disease progression [55, 56]. Additional studies have reported ER stress induction, misfolding of MUC2, defects in protein crosslinking, and reduced glycosylation as features of severe UC [57, 58]. Together, these findings highlight that both structural and functional defects in goblet cells compromise the mucus barrier and contribute to disease.

1.3.2 Goblet cell subsets in inflammation

Recent studies have also drawn attention to changes in specific goblet cell subsets during disease. In active UC, both intercrypt goblet cells (icGCs) and sentinel goblet cells (senGCs) are reduced, likely due to increased autophagy and cell shedding [6, 21]. In IBD more broadly, an imbalance between canonical and non-canonical goblet cells has been reported. Inflammation of the epithelium drives upregulation of *DMBT1*, a marker of non-canonical goblet cells, in mouse models [25]. At the same time, the gene *WFDC2*, a protease inhibitor with antibacterial activity normally associated with non-canonical goblet cells is downregulated [25, 59]. These opposing changes suggest that goblet cell subsets are differentially affected during inflammation, and that their altered function contributes to barrier breakdown. Collectively, these studies point toward a central conclusion that disruption of goblet cell function leads to weakening of the mucus barrier, which in turn exacerbates chronic intestinal inflammation in both humans and mice.

1.3.3 DSS model of colitis

One of the most widely used experimental systems to study colitis in mice is the dextran sodium sulfate (DSS) model. DSS is a cytotoxic compound that damages the mucus barrier, allowing bacteria to translocate to the epithelium and trigger

inflammation. Prolonged exposure to DSS results in rapid onset of colitis and can be lethal. Although the exact mechanism by which DSS induces inflammation is not fully understood, it remains a robust and reproducible model for studying barrier dysfunction.

Importantly, many of the goblet cell alterations observed in human IBD are also reproduced in the DSS model. Crucially, mice lacking Muc2 (*Muc2-/-*) develop severe and rapid colitis when treated with DSS, whereas wild-type (*Muc2+/+*) mice are more resistant [53]. This finding underscores the essential role of Muc2 and other goblet cell-derived proteins in protecting against chemically induced colitis. The DSS model therefore not only provides a tool to study disease mechanisms but also reinforces the central importance of mucus and goblet cells in maintaining intestinal homeostasis.

1.3.4 Bacterial infections

Several bacterial pathogens, including enteropathogenic, enterohaemorrhagic, and enterotoxigenic strains of *E. coli*, *Vibrio cholerae*, *Shigella* spp., *Salmonella* spp., and *Listeria* spp., are well known to infect the small intestine or colon and drive inflammation that often results in diarrhea. For these organisms to colonize the intestinal epithelium, they must first cross the protective mucus barrier. Some bacteria are flagellated and motile, which allows them to move through the mucus layer. Others, such as *Citrobacter rodentium*, the murine counterpart of EHEC and EPEC lack flagella and have evolved alternative strategies to penetrate the barrier. A major mechanism that enables bacteria to cross the mucus is the production of mucolytic enzymes which degrade the mucin gel and open a path to the epithelial surface [60-62].

Certain pathogens also exploit anatomical weak points. For example, *Shigella* and *Salmonella* often target the regions above follicle-associated epithelium, where goblet cell density is lower and mucus protection is reduced[63-65]. The importance of goblet cell-derived mucus in resisting infection is highlighted by studies in mice. In one experiment, loss of *Muc2* in the C57BL/6 strain made animals highly susceptible to *C. rodentium* infection, leading to severe tissue and systemic bacterial burden and ultimately death [66]. These studies emphasize that goblet cells and their secreted mucus are essential for maintaining a sterile barrier and protecting the intestine from pathogenic invasion.

1.3.4.1 Murine model of C. rodentium infection

Citrobacter rodentium is a natural mouse pathogen that has become a widely used model for studying host–pathogen interactions in the colon [67, 68]. Like EHEC and EPEC in humans, *C. rodentium* forms attaching and effacing lesions on the distal colon epithelium [67, 68]. It's understandable infection dynamics and relative ease of handling make it an excellent system to test mucus and goblet cell mediated protective mechanisms. Susceptibility to *C. rodentium* varies between mouse strains and is influenced by factors such as microbiota composition, genetic background, and housing conditions. In C57BL/6 mice infection is usually self-limiting, whereas strains like C3H or AKR develop severe disease characterized by diarrhea, dehydration, and high mortality [69, 70]. Because of this difference, C57BL/6 mice are often suited to study fundamental aspects of goblet cell protection as they allow controlled infection without rapid lethality.

The infection cycle of *C. rodentium* can be divided into four broad stages. After inoculation, a small fraction of bacteria colonize the caecal patch, adapting to the host microenvironment during the establishment phase, which lasts for the first three days [71, 72]. This is followed by the expansion phase, from days 4 to 8, when bacteria migrate from the caecum to the colon, make intimate contact with epithelial cells, and activate virulence genes that drive proliferation and spread further [73]. The next stage is steady colonization, where the pathogen replicates in both colon and caecum while simultaneously being shed via stool [72]. Finally, in susceptible strains, this can progress to severe disease while in resistant strains the infection is gradually cleared.

1.3.4.2 C. rodentium infection strategy

The ability of *C. rodentium* to colonize the intestine depends on a specialized set of genes located in the locus of enterocyte effacement (LEE) pathogenicity island. This region encodes the outer membrane adhesin intimin and its receptor, structural components of the type III secretion system (T3SS), multiple effector proteins, as well as regulators and chaperones required for their function [74].

During caecal colonization, *C. rodentium* senses the host microenvironment and adjusts its expression of LEE genes accordingly. Signals such as bacterial metabolites, temperature, levels of antimicrobial proteins, sulfate availability, and host hormones can all influence this regulation [75-77]. Colonization of distal colonic epithelial cells occurs through the formation of attaching and effacing (A/E) lesions. These lesions involve destruction of the brush border and intimate bacterial attachment, mediated by intimin binding to the translocated intimin receptor (Tir) which the bacterium

delivers into host cells via the T3SS. Throughout the expansion phase, *C. rodentium* injects a wide array of effector proteins into host cells. These effectors manipulate host signaling pathways, cytoskeletal structures, and immune responses, creating a niche that favors bacterial persistence and growth. By altering host cell processes, the pathogen ensures its survival, replication, and dissemination within the colon.

1.3.4.3 Host response to C. rodentium infection

A defining feature of *C. rodentium* infection is the development of transmissible colonic crypt hyperplasia (CCH) [78]. The intimate attachment and replication of the pathogen cause epithelial cell death, which in turn triggers a tissue repair response. This response is marked by an increase in Lgr5+ stem cells at the crypt base, along with the presence of incompletely matured transit amplifying (TA) cells [73, 79]. One of the earliest visible signs of these changes is an increase in crypt length, which becomes evident around six days after infection [73]. To cope with the stress of infection, the host also alters its cell cycle and DNA repair pathways [73]. At the same time, there is a reduction in metabolic processes such as pyruvate, starch, and glucose metabolism, while pathways linked to cholesterol biosynthesis and efflux are magnified possibly to meet the higher energy demands of active infection [80].

Once *C. rodentium* attaches to intestinal epithelial cells (IECs), it triggers a strong inflammatory response. This begins with the activation of pattern recognition receptors including TLR2, NOD2, TLR4, and TLR9. These receptors recruit adaptor molecules such as MyD88 and TRIF, which together drive the production of pro-inflammatory cytokines [81-83]. Inflammasome activation also plays a key role in protection. Caspases 1, 4, and 11 are activated, leading to the release of IL-1β and IL-18 [84-86]. This release depends on inflammasome sensors such as NLRC4 and NLRP3 together with the adaptor ASC and has been shown to be essential for limiting disease severity and aiding clearance of the pathogen [87-89].

Secreted cytokines including IL-6, IL-23, and IL-22 further shape the response by promoting neutrophil infiltration and stimulating the production of antimicrobial peptides such as calprotectin and lipocalin-2 [90, 91]. REG3β can drive IL-17 release from ILC3 cells, while IECs themselves produce IL-33 which is an alarmin that induces secretion of another antimicrobial peptide, REG3γ [92, 93]. Adaptive immunity also contributes. In the early phase of infection, CD4+ T cells dominate with Th22 and Th17 cells producing IL-23 and IL-17, respectively [94, 95]. As infection progresses, the response shifts toward a Th1 profile, characterized by increased IFNγ production [96]. Overall, the host response to *C. rodentium* is a tightly coordinated system. It combines epithelial repair, innate immune activation, adaptive

T cell responses, and goblet cell-mediated mucus clearance, ensuring that the infection is controlled and eventually resolved.

2 Aims

The overall aim of this thesis is to uncover how goblet cells and intestinal mucus barriers influence epithelial protection, and how their disruption creates vulnerabilities to enteric pathogens. In particular, the following papers addressed the specific aims as follows:

Paper 1: To investigate how cellular, molecular, and microbiota-dependent mechanisms regulate the postnatal development of the colonic inner mucus layer, as well as the postnatal development and functional maturation of sentinel goblet cells in the colonic epithelium.

Paper 2: To investigate the contributions of distinct colonic mucus layers and specialized goblet cell subsets to the primary protection of colonic epithelial surface, and to explore how enteric bacterial pathogens manipulate these cells to facilitate colonization and persistence.

Paper 3: To investigate how sentinel goblet cells function as a secondary defensive program, dictating inducible mucus secretion and shaping downstream crypt-intrinsic protective responses when the primary colonic mucus barrier is breached.

Paper 4: To employ *Citrobacter rodentium* as a model pathogen for determining whether disruption of the jejunal mucus barrier enables colonization of an otherwise resistant intestinal niche.

3 Methods

3.1 Animal models

To address the aims of this thesis, we employed a range of animal models that enabled us to investigate the development of mucosal defense, define the relative contributions of distinct GC subtypes to barrier protection, and identify genotype inflicted phenotypic differences against chemical model of colitis, microbial status, and infection that might influence barrier integrity.

In Paper I, we examined the postnatal establishment of the mucus barrier using neonatal rats, together with adult rats and mice. These models provided a developmental understanding of mucus barrier formation, while reporter mice (RedMUC2^{98tr}) and knockout strains (*MyD88*$^{-/-}$, *Nlrp6*$^{-/-}$, and *Duox2* conditional knockouts) offered additional insight into epithelial signaling pathways that influence barrier maturation. In Paper II, we shifted focus to infection-driven disruption of the barrier. Using adult C57BL/6 mice, including *Tgm3*$^{-/-}$ and *Spdef*$^{-/-}$ mutants together with Muc2-YFP reporter animals, we were able to follow the sequence of events leading to barrier breakdown during *Citrobacter rodentium* infection. These models also revealed how specific GC subtypes contribute to frontline defense against infection. In Paper III, we investigated secondary defense mechanisms mediated by senGCs. To do this, we generated GC–specific Nlrp6 knockouts by crossing Muc2-iCre mice with *Nlrp6*$^{fl/fl}$ animals. This model system allowed us to directly test the role of senGCs in maintaining crypt sterility and preserving spatial separation between bacteria and the epithelium during infection. Across all studies, experimental comparisons were made using co-housed littermate controls unless otherwise specified, ensuring that observed differences truly reflected biological variation.

3.2 Biological and chemical agents of colitogenesis

In paper II and III, we used *Citrobacter rodentium* infection as a model to deliberately disrupt the intestinal barrier and evaluate GC responses. All infections were performed with either streptomycin-resistant *C. rodentium* DBS100 strain or the variant that lacked T3SS effector protein EspF. For colonic whole-mount analyses, we employed a GFP-expressing variant of this strain (DBS100-GFP), generously provided by Bruce A. Vallance. Bacteria were grown overnight in LB broth at 37 °C, and mice were orally gavaged with 200 µL of culture containing approximately 1–4 × 10^9 CFU. At selected time points after infection, animals were sacrificed, and distal colonic tissue was collected. Luminal contents and tissue samples were

processed separately to assess bacterial burden, and colonic whole-mounts were prepared to visualize bacterial localization relative to the mucus barrier.

In Paper III, we evaluated the protective role of senGCs using a chemically induced model of colitis [15, 97]. We administered 3% dextran sodium sulfate (DSS) in the drinking water to trigger barrier disruption and monitored body weight daily throughout the treatment period. On day 8, we sacrificed the mice and collected tissues for analysis. We evaluated disease severity by combining macroscopic and microscopic readouts. Colon length and spleen weight provided gross indicators of inflammation, while histopathological analysis of distal colon sections offered a more detailed view. Histology scoring covered key features of tissue damage, including inflammation, crypt architecture, GC depletion, regeneration, and muscle thickening. By including both *C. rodentium* infection with DSS-induced colitis, we were able to analyse GC-mediated defense under two distinct but potent models of barrier disruption, thereby strengthening our understanding of GC protective functions.

3.3 Ex vivo mucus analysis:

The analysis of the mucus layer associated with the colonic epithelial surface has evolved considerably over time. While early studies relied on laborious *in vivo* measurements of mucus thickness in rats [98], more recent approaches have used histological staining and interpretation of mucus thickness from tissue sections [99]. However, histological methods introduce technical artefacts that can yield variable results, primarily due to the choice of fixatives which often cause shrinkage of IML and thereby distort measurements. Therefore, it is important not to rely solely on histological analysis for quantifying mucus layer properties. This limitation was previously highlighted by Volk et al., who also presented a viable alternative for measuring mucus thickness *ex vivo*, building on the original method described by Gustafsson et al [17, 100].

In our experiments, colonic tissue was extracted from mice and mounted in a custom-designed horizontal chamber equipped for liquid perfusion, with the mucosal surface oriented upward. This setup enabled direct visualization of the tissue by confocal microscopy, or measurement of live mucus growth kinetics using a glass capillary needle under a stereomicroscope. Importantly, this *ex vivo* method allows detection of rapid changes in mucus properties and evaluation of growth kinetics in response to agonists or inhibitors, thereby providing a powerful means to analyze the highly dynamic behavior of goblet cells and the dependent mucus layer during enteric bacterial infections. Therefore, I extensively adapted and applied this state-of-the-art approach to address key questions outlined in the aims section (Figure 4).

3.3.1 Ex vivo mucus growth kinetics

Using the described system, colonic tissue was mounted and overlaid with black latex microbeads (bacteria-sized) to visualize the mucus surface. A glass capillary needle was then used to measure the distance between the epithelial layer and top of the mucus surface, providing an accurate assessment of the mucus layer intimately attached to the epithelium at t = 0. Baseline mucus secretion was subsequently quantified by repeating these measurements every 15 minutes for one hour. To assess how chemically induced colitis or infection alters this process, we combined this experimental setup with DSS-induced colitis (Paper I) and *C. rodentium* infection (Papers II & III), enabling simultaneous measurement of the impact of these challenge models on mucus growth kinetics. Previous studies have shown that senGCs respond to bacterial MAMPs through compound mucus secretion, a process initiated by endocytosis of MAMPs, TLR signaling, and activation of the *Nlrp6* inflammasome, as described in the introduction [35]. Leveraging this system, we combined MAMP stimulation with endocytosis inhibitors to detect, confirm or quantify senGC maturation (Paper I) and activity (Paper III). Furthermore, we addressed the impact of infection on senGC frequency in Paper III by integrating ex vivo mucus measurements with bacterial MAMP treatments (Figure 4).

While our system provides a robust model to quantify mucus growth kinetics, it presented some challenges when analyzing tissues from *C. rodentium*–infected animals. Infection disrupts the integrity of the IML and often causes its collapse, making it difficult to detect changes in mucus growth dynamics. In our experiments, at 7 days post-infection (dpi), tissues failed to mount a secretory response upon P3CSK4 stimulation. However, subsequent histological analysis revealed cavitations extending from deep crypt regions, suggesting that an intact IML may be required to reliably measure mucus secreted by epithelial goblet cells. In some aspects, these findings may also reflect the impact of infection-induced alterations in post-secretory mucus processing by Clca1 or Tgm3, which can influence the integrity, cohesiveness, and barrier tightness of the secreted mucus layer[12, 101-103]. Moreover, infection imposes additional stress on the epithelial surface, increasing cell fragility and susceptibility to death, which makes maintaining viable tissue for 60 minutes under *ex vivo* conditions a significant challenge. This factor could also contribute to the poor MAMP-induced mucus growth rate quantified, even though the perfusion chambers and temperature control systems were designed to mimic in vivo conditions by providing appropriate temperature and nutritional support. These limitations must therefore be carefully considered when applying this method in the context of infection or other challenge models that may cause disruption of IML.

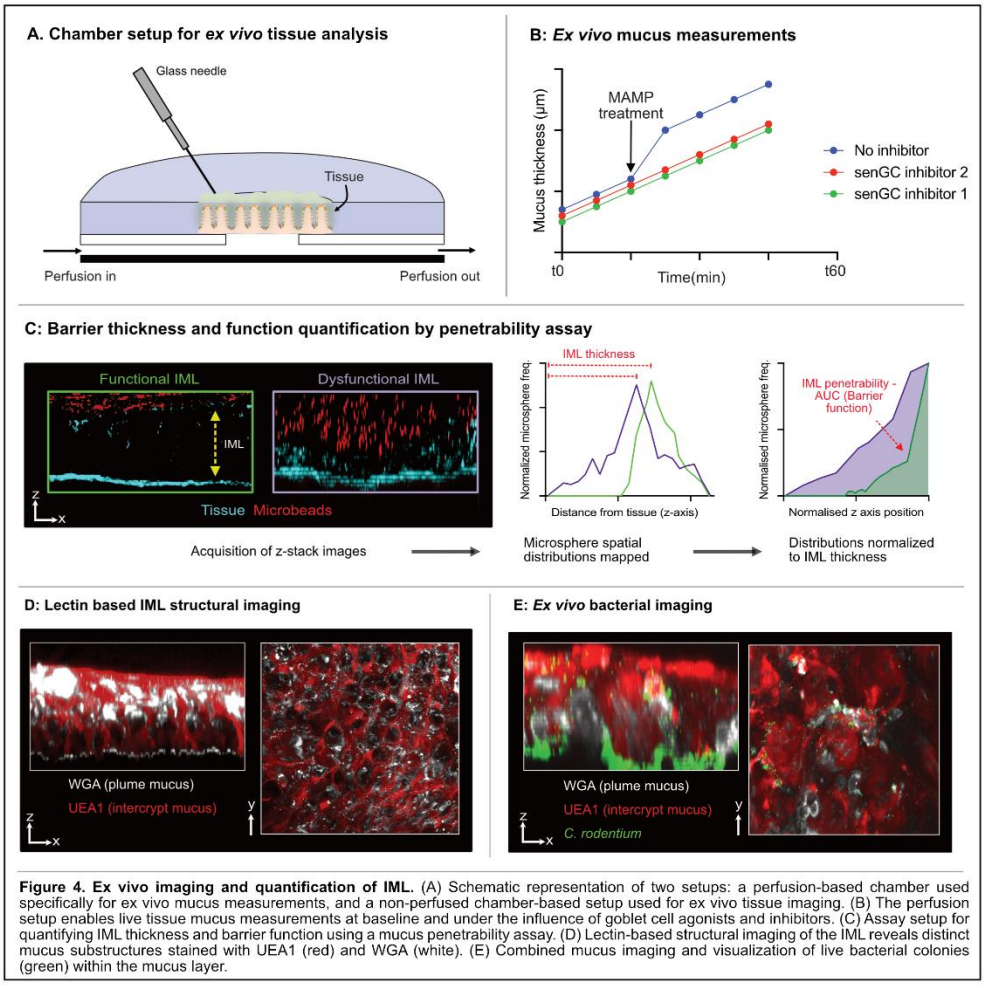

Figure 4. Ex vivo imaging and quantification of IML. (A) Schematic representation of two setups: a perfusion-based chamber used specifically for ex vivo mucus measurements, and a non-perfused chamber-based setup used for ex vivo tissue imaging. (B) The perfusion setup enables live tissue mucus measurements at baseline and under the influence of goblet cell agonists and inhibitors. (C) Assay setup for quantifying IML thickness and barrier function using a mucus penetrability assay. (D) Lectin-based structural imaging of the IML reveals distinct mucus substructures stained with UEA1 (red) and WGA (white). (E) Combined mucus imaging and visualization of live bacterial colonies (green) within the mucus layer.

3.3.2 Ex vivo mucus penetrability assay

In this thesis, I have modified the *ex vivo* mucus analysis setup to evaluate the barrier function of the colonic mucus layer. Specifically, to assess the penetrability of the distal colonic IML, fluorescent microbeads (1 µm diameter) were applied to the apical surface of freshly mounted tissue placed in the pre-chilled perfusion chamber. After allowing the beads to settle onto the mucus surface, excess beads were gently rinsed off, and the tissue was imaged using confocal z-stack microscopy. The vertical positions of both the epithelial surface and individual microbeads were extracted to determine mucus thickness and analyze the spatial distribution of beads within the mucus layer. To quantify penetrability, frequency curves representing bead depth

were generated and normalized to account for differences in total bead counts and variations in mucus surface position across samples. The area under each normalized curve was calculated to provide a comparative measure of mucus penetrability, enabling assessment of barrier integrity under different experimental conditions (Figure 4).

In Paper I, we used neonatal rats to track the postnatal development of a functional colonic mucus barrier. In Paper II, this *ex vivo* method was applied to study the impact of *C. rodentium* infection on the colonic mucus barrier, specifically by quantifying changes in IML thickness and penetrability. In Paper III, we evaluated the protective role of senGCs during *C. rodentium* infection, with a focus on their contribution to maintaining barrier integrity. Across these studies, the adapted *ex vivo* mucus penetrability analysis provided a powerful platform to assess dynamic changes in mucus barrier function under both developmental and pathological conditions.

3.3.3 Ex vivo mucus-bacteria imaging

In this thesis, we adapted an ex vivo confocal imaging approach to study mucus–bacteria interactions at the colonic surface during Citrobacter rodentium infection. Tissue was harvested from animals infected with GFP-expressing C. rodentium DBS100 and mounted with the mucosal surface exposed. Dual lectin staining was used to resolve distinct mucus compartments: Rhodamine-conjugated WGA labeled the epithelial surface and crypt plume mucus, while Alexa 647-conjugated UEA-1 highlighted intercrypt mucus (Figure 4).

In Paper II, this method favoured high-resolution visualization of bacteria, mucus architecture, and host tissue during infectious stress, allowing us to track spatial changes in mucus substructure across the infection cycle. A key advantage of this approach was its ability to simultaneously visualize distinct mucus substructures allowing us to track regional changes to both intercrypt and crypt plume mucus during infection. This structural resolution is difficult to achieve with conventional histology, underscoring the benefits of this technique towards understanding localized barrier dynamics. In Paper III, we extended this imaging strategy to evaluate the role of senGCs in upholding barrier integrity and spatial separation between bacteria and the epithelial surface when there is an active invasion of the IML. The ability to simultaneously visualize bacterial localization within mucus secreted from upper crypt regions provided a powerful means to assess senGC-mediated defense mechanisms in an *ex vivo* setting.

3.3.4 Ex vivo faecal mucus layer imaging

In Papers II and III, we modified the *ex vivo* imaging setup to examine the mucus layer that surrounds faecal pellets, moving beyond the conventional focus on inner and outer mucus layers (Figure 5). Earlier approaches primarily assessed mucus directly attached to the tissue, often overlooking the outer layer that is actively displaced and wrapped around the stool during transit [18, 66]. This faecal-associated mucus compartment is rich in luminal bacteria and contains microbes shed from tissue-adherent mucus forming a dynamic interface between host and microbiota [19]. Therefore, to capture this layer, stool samples from control and *C. rodentium* GFP DBS100-infected mice were collected during colon dissection and placed in a custom imaging chamber designed to stabilize and immobilize the pellet. The stool associated mucus was stained with Rhodamine-conjugated WGA and Alexa 647-conjugated UEA-1 which allowed us to distinguish different mucus components. Furthermore, confocal z-stack imaging provided a detailed view of stool encapsulated mucus and *C. rodentium* distribution within the stool. Image analysis using Imaris enabled quantification of mucus volume, dimensions, and thickness. Overall, this approach offered a more physiologically relevant perspective on spatial changes to mucus organization during infection by visualizing the spatial structure and microbial content of the faecal-encapsulating mucus layer. Additionally, we could assess how infection influences mucus shedding, microbial clearance, and barrier dynamics in a compartment that links luminal and tissue-associated environments.

3.4 Bulk RNA sequencing

In Paper I, we used bulk RNA sequencing to explore how the expression of genes linked to GC functioning, mucus processing, activity changed in the colon with respect to age and simultaneous changes occurring in the mucus barrier. High-quality RNA was extracted from colonic tissues of neonatal (P9–P22), weaned (P24–P33), and adult (>P100) Wistar rats under both ConvR and GF conditions. For certain comparisons, we used these new datasets in conjunction with previously published bulk and single-cell RNA-seq data from sorted goblet cells and enterocytes, to allow us to place our findings in a broader systemic context. Differential expression and correlation analyses then highlighted gene programs that shift with age, microbial presence or differences, while single-cell data helped us identify goblet cell subtypes and their relevance to barrier maturation.

In Paper II, we wanted to investigate GC transcriptome changes that might influence GC associated protective function during *C. rodentium* infection. Additionally, we

aimed to determine whether the absence of the type III secretion system effector EspF in *C. rodentium* would elicit a GC transcriptional program distinct from that induced

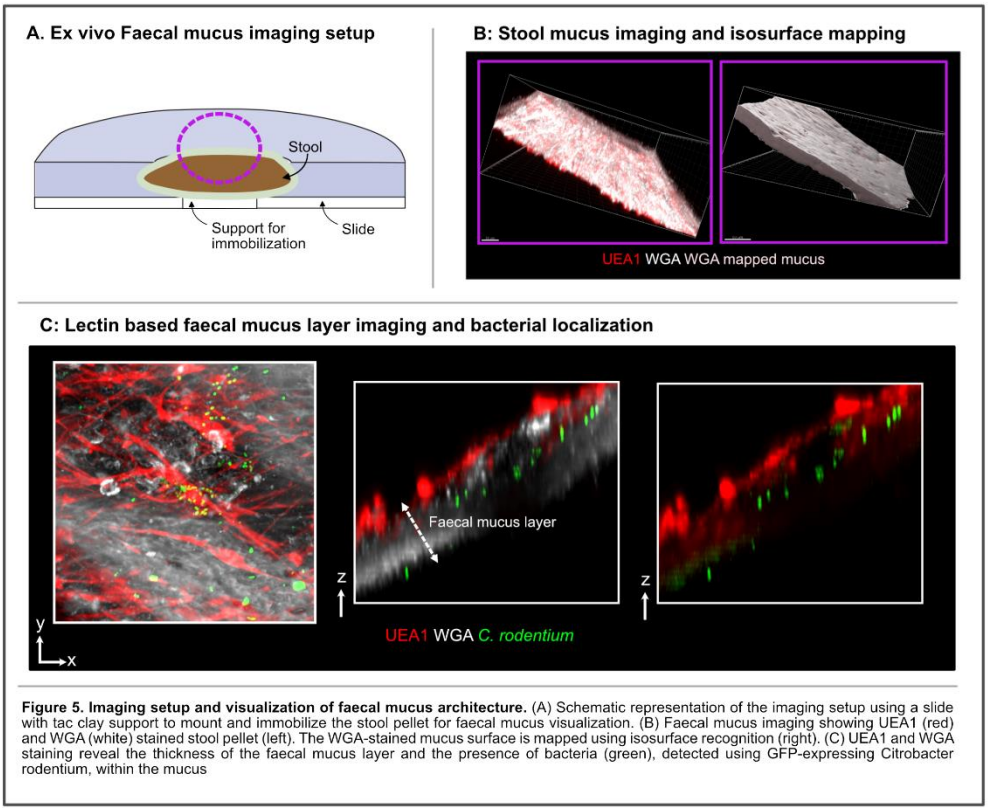

Figure 5. Imaging setup and visualization of faecal mucus architecture. (A) Schematic representation of the imaging setup using a slide with tac clay support to mount and immobilize the stool pellet for faecal mucus visualization. (B) Faecal mucus imaging showing UEA1 (red) and WGA (white) stained stool pellet (left). The WGA-stained mucus surface is mapped using isosurface recognition (right). (C) UEA1 and WGA staining reveal the thickness of the faecal mucus layer and the presence of bacteria (green), detected using GFP-expressing Citrobacter rodentium, within the mucus

by wild-type infection. To do this, we infected Muc2YFP mice with either WT *C rodentium* or the variant that lacked T3SS effector protein EspF and then sorted YFP^{+} goblet cells and neighboring epithelial cells by flow cytometry. RNA was extracted directly from these purified populations and sequenced to reveal alterations in cell-type–specific transcriptional programs between WT and the EspF knockout *C. rodentium* infection.

In Paper III, we used bulk RNA sequencing of distal colonic epithelial tissue from *C. rodentium* infected $Nlrp6^{fl/fl}$ and $Nlrp6^{\Delta GC}$ mice to obtain information on broad transcriptional programs and assess how the absence of senGCs shapes epithelial responses. Since we observed that female $Nlrp6^{\Delta GC}$ animals were more susceptible to *C. rodentium* infection, sex was explicitly included as a biological variable in the

experimental design. By sampling both males and females at baseline and across multiple infection time points, we could distinguish genotype-dependent effects from infection-driven changes and evaluate how sex influenced these transcriptional trajectories.

3.5 Ethical considerations

All experiments were carried out under approved ethical permits and with close attention to animal welfare. In Paper I, we included both neonatal and adult Wistar rats to study developmental aspects of the mucus barrier. All rats were obtained from an approved commercial breeder and housed under specific pathogen-free conditions with free access to food and water. We monitored the animals daily to ensure their wellbeing, and all procedures were carried out under valid ethical permits. Neonatal rats were euthanized by decapitation, while adult rats were anesthetized with isoflurane and sacrificed by cervical dislocation. These procedures were chosen to minimize suffering and were consistent with established humane endpoints in our approved ethical permits. In Papers II and III, where we performed *C. rodentium* infection studies, we applied a humane endpoint of 15% body weight loss. Infections were self-limiting, and experiments were conducted for up to 28 days by which time the animals had typically cleared the pathogen. In Papers I and III, we used 3% DSS in drinking water to induce colitis in WT and *Nlrp6*$^{\Delta GC}$ mice. Because DSS is a potent colitogenic agent that can cause rapid weight loss, we monitored the animals closely and sacrificed them once they reached a 15% reduction in their starting body weight.

4 RESULTS AND DISCUSSION

4.1 Paper I: Functional development and maturation of senGCs

The colonic environment is highly dynamic, constantly exposed to both commensal microbes and any potential pathogens [50, 66]. Protection against these challenges is provided by the inner mucus layer, which normally remains free of bacteria and offers a critical physical barrier [104]. While the importance of goblet cells and their secretion of Muc2 in establishing and maintaining this barrier is well recognized [53, 105], the natural developmental sequence and epithelial factors that prime its early formation remain poorly understood. Within this context, a specialized frontline population of senGCs reside at crypt entrances, where they have been proposed to sense bacteria and rapidly expel mucus [35]. However, whether the senGC system develops constitutively or requires microbial induction during the postnatal period has not been defined. Addressing this question is essential for understanding the neonatal events that shape senGC development and the cues that establish their protective role. In this study, we therefore tracked the sequence of events underlying mucus barrier formation and senGC maturation in relation to postnatal microbial colonization, noting that senGCs are absent in adult germ-free mice but emerge during the natural window of microbial exposure.

4.1.1 Microbiota-driven induction of GC functional maturation

During the crucial neonatal window when colonic protective systems are functionally developing, we observed an age-dependent induction of core mucus components (Clca1, Fcgbp, Zg16) and transcriptional regulators of goblet cell secretion (Atoh1, Gfi1, Foxa3), but only in conventionally raised (ConvR) mice. This indicated that postnatal goblet cell functional maturation is microbiota-driven. In addition, we found that microbiota was essential for regulating mucin glycosylation (e.g., Slc35b3, Slc35a2, Galnt4, C1galt1) and intracellular cargo transport (Stx5a, Copa, Cog2), further cementing its role in shaping the colonic GC functional landscape. Of importance, most of these differences were evident only in the postnatal ConvR vs GF comparisons and were largely lost in adult comparisons. This likely reflects that adult GF mice, through dietary exposure to dead microbial components can partially stimulate GC responses and thereby blur potential differences.

4.1.2 Complex microbial communities are required for senGC maturation

To test whether different microbial exposures influenced senGC maturation, we conventionalized germ-free (GF) mice with a complete microbiota transfer (ConvD) or monocolonized them with *Bacteroides fragilis*, since *Bacteroides* species were enriched in microbiota samples from senGC^{+} neonates (P15) compared to senGC^{-} neonates (P5). We found that conventionalization with a full microbiota for three weeks shifted the secretory program toward a senGC-dependent mode, whereas monocolonization with *B. fragilis* failed to do so. These findings indicate that simple exposure to bacteria is insufficient to drive senGC induction, and that complex microbial communities and their associated signaling are required. While we cannot exclude the possibility that other individual species may contribute*, B. fragilis* alone was not sufficient to promote senGC-dependent secretion.

4.1.3 Innate immune signaling pathways in senGC-independent secretion

We also examined the role of innate immune signaling molecules in senGC maturation. During the first two postnatal weeks, neonatal colonic tissue responses to bacterial MAMPs followed a senGC-independent secretory pathway. Histological analysis revealed deep crypt cavitations consistent with fluid secretion, which may serve to flush bacteria from crypts during this vulnerable period when the mucus barrier is still stabilizing. Interestingly, adult GF mice also exhibited a senGC-independent secretory response that was critically dependent on *MyD88*. Using senGC pathway inhibitors and GF mice lacking *Nlrp6*, we further showed that senGC-independent secretion was not reliant on endocytosis, caspase activation, or the *Nlrp6* inflammasome. Finally, when Nlrp6$^{-/-}$ mice were conventionalized as adults, they retained a senGC-independent secretory response, in contrast to WT mice which transitioned to senGC-dependent secretion after four weeks. By comparison, ConvR Nlrp6$^{-/-}$ animals lacked both senGC-dependent and senGC-independent programs. These findings suggest that the induction of a senGC-independent pathway in Nlrp6$^{-/-}$ mice is shaped by colonization timing and microbial context, rather than representing the idea that a stable compensatory mechanism is developed because of *Nlrp6* deficiency in the postnatal window.

4.1.4 Noncanonical GCs and remodeling of the mucus proteome

The emergence of robust, *Nlrp6* and endocytosis-dependent senGC mucus secretion coincides with key changes in IML composition and barrier properties, including a marked increase in regulatory proteins such as Tgm3, which stabilizes and crosslinks secreted mucins to reinforce the structural integrity of the developing barrier [12].

Proteomic analyses across the senGC developmental window revealed the accumulation of a distinct set of mucus proteins with signatures indicative of noncanonical GC origin. These included Tgm3 and Lgals3, both previously implicated in mucosal defense [12, 106], as well as proteases such as Mep1a, Ctsb, Ctsd, and Ctss [107], whose potential roles may extend to antimicrobial or barrier-modulating functions. Importantly, these proteins were comparatively minimally expressed by canonical GCs, suggesting that noncanonical GCs make unique contributions to mucus composition and to the establishment and refinement of the IML barrier during the postnatal period. The coordinated maturation of senGCs in this window might indicate a broader principle: that neonatal mucosa is primed by early microbial exposure not simply to establish a static barrier, but to dynamically regulate barrier properties through diverse GC cell types with distinct secretory contributions.

4.1.5 Duox2 as a mechanistic driver of senGC activation

We further show that functional activation of the senGC secretory pathway is likely supported by postnatal induction of *Duox2 expression*, in line with prior evidence linking *Duox2* to microbiota-regulated epithelial immune responses [108, 109]. Histochemical and immunofluorescence analyses localized Duox2 protein in upper crypt GCs to intracellular compartments consistent with the ER and Golgi apparatus, sites compatible with both secretory cargo processing and the trafficking of endocytosed material [110]. These observations reinforce our earlier findings that senGC activation is triggered downstream of MAMP sensing, endocytosis, and TLR/MyD88 signaling, with Duox2-mediated ROS synthesis acting upstream of Nlrp6 inflammasome assembly as an obligate step before mucus exocytosis [35]. The dependency of this pathway on microbiota-driven *Duox2* expression provides a mechanistic explanation for why functional senGCs fail to develop in GF environments, even though other aspects of GC biology remain partially intact.

In summary, paper I outlines a two-level model for maturation of postnatal colonic epithelial defense. In the neonatal window before senGCs are fully functional, MAMP challenge elicits a MyD88-dependent fluid secretory response, likely serving as a provisional mechanism to flush microbes from crypt niches while the mucus barrier is still consolidating. With progressive microbial colonization, Duox2 induction primes an endocytosis and *Nlrp6*-dependent pathway in senGCs, enabling rapid ROS generation, inflammasome activation, and robust mucus discharge. In parallel, functional maturation of upper crypt noncanonical GCs and the enrichment of their characteristic protein signatures in the mucus proteome actively shape the IML, providing qualitative enhancements beyond canonical GC activity. Together, these intersecting processes ensure that the developing colon adapts its barrier properties to

evolving microbial exposure, establishing both tolerance to commensals and effective defense against pathogens.

4.2 Paper II: Functional disruption of the colonic mucus barrier during *C. rodentium* infection

The colonic mucus barrier is a critical first line of defense with the IML preventing direct bacterial contact with the epithelium [19, 104]. Previous studies established that *C. rodentium* can colonize the colonic epithelium and induce mucus secretion from GCs [66, 105], but how this pathogen destabilizes the mucus barrier and whether it selectively targets specialized GC subsets or GC associated protective mechanisms remained unclear. In particular, the series of events that drive mucus barrier loss and pathogen persistence in relation to GC functioning had not been defined. Addressing this gap, we investigated how *C. rodentium* infection remodels the colonic mucus barriers and GC protective response, and identified bacterial and host factors that determine barrier integrity and pathogen clearance

4.2.1 Barrier destabilization and pathogen shedding

We found that *C. rodentium* infection rapidly thinned the IML, and impacted barrier function by increasing penetrability, and observed a transient increase in FML with visible bacterial clusters. Correlation analyses revealed that mucus penetrability and IML thickness tightly associated to luminal bacterial accumulation, whereas tissue-associated burdens remained broadly stable. These findings advance earlier observations of bacteria within mucus by showing that bacterial signals within the mucus layer likely reflect outward shedding of pathogens into the FML as the IML destabilizes, rather than active penetration of an intact barrier [66]. Additionally, the persistence of bacterial clusters at the periphery of the FML indicated a pattern of active removal of bacteria supported by an infection-stage dependent increase in visible luminal bacterial clusters.

In our results, the transient expansion of the FML, despite impaired mucus replenishment from the distal colon, likely reflects a compensatory secretory response originating from more proximal regions of the gut. This pattern suggests a systemically orchestrated host response to infection, rather than a localized epithelial response from the colonized segments of the colon. Taken together, these findings indicate that the distinction between IML and FML is not just structural, but both layers are dynamically modulated and interconnected during infection by host and pathogen interactions.

4.2.2 GC dynamics and proliferative responses

Using the RedMUC2^{98tr} reporter strain, we showed that infection induced a marked proliferative expansion of Ki67^{+} GCs without altering overall Muc2 expression. This indicated that IML disruption and loss of barrier protection is not due to GC loss or reduced Muc2 expression but rather reflected targeted architectural breakdown of the mucus barrier via other means. The correlation between pathogen burden and GC proliferation suggested that *C. rodentium* drives epithelial hyperplasia, which may transiently sustain mucus output even as barrier quality deteriorates. These findings indicate that although infection drives expansion of Ki67+ GCs, this response does not maintain an intact IML. Instead, barrier disruption reflects architectural breakdown of the mucus layer despite preserved GC numbers and Muc2 expression.

4.2.3 Spdef-dependent icGCs as critical defenders

A central novel finding of this study is the identification of Spdef-dependent icGCs as indispensable for early defense and timely clearance of *C. rodentium*. *Spdef*-deficient mice exhibited impaired control of infection, delayed pathogen clearance, and increased crypt infiltration, confirming the role of icGCs and the intercrypt mucus they produce in protection against enteric bacterial infection. In contrast, deletion of Tgm3, despite being a stabilizing agent of crypt plume mucus, did not alter infection kinetics highlighting that intercrypt mucus is perhaps the more critical barrier component during early infection. Imaging confirmed that icGCs and their intercrypt mucus lattice were selectively depleted during peak infection, while crypt plume mucus was still detectable but reduced compared to normal, consistent with its dependence on intercrypt mucus for stabilization. This created permissive niches for bacterial persistence. Importantly, this suggested that crypt plume mucus alone cannot safeguard the epithelium when the intercrypt matrix is compromised. Our findings build on earlier studies by identifying icGCs the critical mucus-producing subset targeted during infection [21].

4.2.4 EspF-driven depletion and transcriptional remodeling of GCs

Mechanistically, we identified the bacterial type III secretion system (T3SS) as essential for icGC depletion. Infection with *ΔescN* mutants preserved icGC numbers, confirming the requirement for a functional T3SS. Loss of EspF moderately prevented icGC depletion, suggesting that EspF is a likely effector contributing to this process. Transcriptomic profiling revealed that EspF profoundly reprogrammed the GC transcriptome: WT infection downregulated lineage factors such as *Atoh1* and induced sterol pathways, while *ΔespF* infection preserved *Atoh1* and upregulated *Spdef*,

consistent with improved GC survival which likely contributes to maintenance of intercrypt mucus. These findings extend earlier work on EspF's role in disrupting epithelial junctions by showing that it also directly undermines goblet cell survival and function through transcriptional and metabolic reprogramming [111].

Together, our results define a sequence of events in which *C. rodentium* colonization destabilizes the IML, leading to mucus thinning, increased penetrability, and enhanced bacterial shedding. This barrier failure is compounded by EspF-driven depletion of Spdef-dependent icGCs, which dismantles the intercrypt mucus lattice and permits epithelial access. While crypt plume mucus remains partially intact, it is insufficient to compensate for icGC loss, positioning icGCs as the critical barrier subset. By linking EspF bacterial effector activity to targeted depletion and transcriptional remodeling of icGCs, this study advances previous models of infection by showing that pathogens exploit specialized GC subsets to undermine barrier integrity.

4.3 Paper III: The senGCs govern crypt-specific defense against enteric pathogens

In Paper II, we investigated the primary mucus barriers that dictate colonic defense (IML, FML) and defined the dynamic structural changes to these layers in the event of infection. While these layers collectively restricted microbial access, we also observed that protection of the epithelial surface and crypt spaces is not fixed, as localized breaches of the IML permitted bacterial contact with the epithelium and further enabled crypt invasion. These findings raised the question of whether additional, more specialized GC subsets contribute to safeguarding the vulnerable epithelial niches in such scenarios. In Paper III, we therefore explored the role of senGCs that are positioned at crypt entrances and are hypothesized to sense microbial products and trigger rapid mucus secretion in response to crypt invasion; however, their *in vivo* function remained speculative due to the absence of a targeted knockout model. To address this gap, we generated a GC-specific *Nlrp6* knockout ($Nlrp6^{\Delta GC}$) to selectively disable senGC function, enabling us to test their contribution to protection of the crypt spaces through coordinated functioning of the crypt residing GCs.

4.3.1 Sex-specific differences in susceptibility and underlying mechanisms

We tested whether senGC deficiency altered susceptibility to barrier disruption by both chemical and microbial challenge models. In the DSS colitis model, female $Nlrp6^{\Delta GC}$ mice exhibited significantly greater disease severity than female controls, as

reflected by reduced colon length and higher histological scores. Male *Nlrp6*$^{\Delta GC}$ mice showed only modest differences from wild-type. A similar pattern was observed during *C. rodentium* infection. At 3 and 7 dpi, female *Nlrp6*$^{\Delta GC}$ animals carried significantly higher mucosal bacterial burdens and displayed marked crypt invasion on confocal imaging, whereas male *Nlrp6*$^{\Delta GC}$ mice maintained bacterial loads comparable to controls across all time points. These findings established a consistent female bias in susceptibility, which prompted further investigation into the molecular and immunological basis of this difference.

We therefore performed bulk RNA sequencing of colonic epithelial cells. At baseline, female *Nlrp6*$^{\Delta GC}$ mice demonstrated upregulation of immunoglobulin genes (*Igha, Igkv8-28, Igkv7-33*) and GC–associated transcripts (*Reg4, Zg16, Ang4*), suggesting a primed mucosal state even in the absence of infection. Male *Nlrp6*$^{\Delta GC}$ mice, by contrast, showed minimal baseline transcriptional alterations. Upon infection, wild-type females mounted a rapid immune transcriptional program enriched for "immune system process" pathways, whereas senGC-deficient females instead showed enrichment of "response to bacterium" pathways, consistent with a compensatory response to elevated pathogen burden. In males, genotype-specific differences were less pronounced, with wild-type animals showing stronger enrichment of defense and inflammasome-related pathways at later stages perhaps driven by activation of senGCs. These data indicate that senGCs are required for the accelerated immune transcriptional response observed in females, and that their absence shifts the epithelial program toward a less effective bacterial stress-response pathways.

Because these transcriptional changes suggested altered immune coordination, we next examined immune cell populations in the lamina propria. At steady state, *Nlrp6*$^{\Delta GC}$ mice of both sexes exhibited reduced neutrophil frequencies compared to controls. During infection, wild-type animals mounted a robust Th17 response, characterized by expansion of *Rorγt*$^{+}$ CD4^{+} T cells, whereas this response was absent in senGC-deficient mice. Instead, female *Nlrp6*$^{\Delta GC}$ animals displayed a compensatory rise in cytotoxic CD8^{+} T cells, a shift not observed in males. These immune alterations complement the transcriptomic findings: loss of senGCs blunts the Th17 axis and forces reliance on an alternative, pro-inflammatory CD8 T cell response.

4.3.2 Dynamic reprogramming and renewed functions of senGCs during infection

To investigate whether senGCs themselves undergo dynamic changes during infection, we examined *Nlrp6* expression in colonic tissue over the course of *C. rodentium* challenge. In wild-type animals, infection induced a marked expansion of

Nlrp6^{+} GCs within the deep crypt regions, in contrast to their predominantly upper-crypt localization at steady state. This redistribution was accompanied by evidence of secretory activity, including cavitation of deep-crypt GCs following stimulation with microbial ligands. These findings indicate that senGCs are not static sentinels but are dynamically reprogrammed during infection to reinforce crypt-level protection through inducible mucus secretion.

Further, the functional consequences of senGC deficiency underscored this role. *Nlrp6*$^{\Delta GC}$ mice exhibited a significantly reduced baseline mucus secretory rate during infection, suggesting that senGCs contribute not only to inducible responses but also to the maintenance of steady-state mucus output under stress. This impairment was accompanied by an aggravated loss of mucin-filled goblet cells as early as 3 dpi, a phenotype not observed in wild-type animals until later at 10 dpi. This early depletion of GCs in senGC-deficient mice likely compromises the capacity to sustain the mucus barrier during infection.

Consistent with this, bead penetrability assays revealed a moderate trend toward increased IML permeability in *Nlrp6*$^{\Delta GC}$ mice at 3 dpi. Although not statistically significant, this early barrier weakness coincided with the loss of GCs and the absence of *Nlrp6*$^{+}$ deep-crypt GCs, suggesting that both factors may act in concert to permit bacterial access to crypt spaces. Thus, the combination of dysfunctional senGCs, accelerated GC loss, reduced baseline mucus secretion, and failure to expand *Nlrp6*$^{+}$ GCs at the crypt base may provide a mechanistic explanation for the enhanced crypt infiltration observed in senGC-deficient mice.

Together, these results reveal that senGCs adopt a renewed role during infection: beyond their conventional function as microbial sensors at the crypt entrance, during infection, they expand into the deep crypts, sustain baseline mucus secretion, and preserve GC numbers. This dynamic reprogramming ensures that the crypt intrinsic secondary mucosal defense is sustained under infectious stress. In the absence of senGCs, these protective adaptations fail, leading to early barrier compromise and increased pathogen access to crypt niches.

4.4 Paper IV: Jejunal mucus barrier disruption enables ectopic *C. rodentium* infection of the region

In Paper IV, we demonstrated that a Western-style diet (WSD) destabilizes small intestinal homeostasis in a regio-specific manner. Our initial analyses showed that 7 days of WSD feeding, but not 3 days, markedly increased the penetrability of the jejunal (SI5) mucus layer compared with chow-fed controls. This defect was

mechanistically linked to enhanced activity of transglutaminase-2 (Tgm2), which promoted cross-linking of Muc2 polymers. The resulting aggregates failed to expand into the intervillus spaces, leaving gaps in the protective layer and thereby compromising barrier function independently of changes in the core mucus proteome.

To assess the biological consequences of this barrier failure, we challenged mice with *Citrobacter rodentium*. In wild-type animals receiving chow diet, infection at 4 dpi was largely confined to the distal colon, with high luminal burdens but negligible colonization of the small intestine and no dissemination to draining lymph nodes. In contrast, mice exposed to 7 days of WSD prior to infection exhibited a pronounced increase in jejunal mucosal colonization, with bacteria detected between villi and in close association with the epithelium. Importantly, viable *C. rodentium* was recovered almost exclusively from the small intestine–draining mesenteric lymph nodes, indicating that loss of the jejunal barrier permitted bacterial translocation. A similar outcome was observed in Muc2-deficient mice, confirming that disruption of the mucus layer alone is sufficient to allow colonization of this normally resistant region. Overall, we showed that disruption of the jejunal mucus barrier by WSD and allows ectopic colonization of the small intestine by *C. rodentium.*

5 Conclusions and future perspectives

In **Paper I**, we have shown that the postnatal maturation of senGCs and the establishment of a functional colonic mucus barrier are critically dependent on microbial colonization. Early in life, when senGCs are not yet fully active, the colon mounts a senGC-independent fluid secretory response likely as a provisional defense. With progressive microbial exposure, *Duox2* induction enable senGC-mediated mucus release, while noncanonical goblet cells contribute distinct proteins that remodel and strengthen the inner mucus layer. Together, these findings define a two-tiered developmental program in which the neonatal colon transitions from temporary protective mechanisms to a mature, microbiota-primed barrier.

While our findings establish that the transition from senGC-independent to senGC-dependent secretion occurs around postnatal day 5-15 and in ConvD mice after three weeks, coinciding with microbial composition changes and Duox2 signaling, the upstream cues that initiate this functional switch remain unresolved. It is not yet clear whether defined microbial metabolites act as the primary triggers for Duox2 induction and senGC activation or whether more complex community interactions are required. Addressing this question will be critical for understanding the pathways and factors that drive the developmental handover between provisional and mature mucus secretory programs. Moreover, the maturation of other goblet cell subsets such as icGCs, remains poorly characterized, despite their protective role in infection and colitis. Determining when icGCs acquire full functionality, what microbial or host factors govern their development, and how they integrate with senGCs to shape the mucus barrier will provide important insights into the layered architecture of epithelial defense during early life.

In **Paper II**, we showed the sequence of events during infection that leads to barrier disruption and bacterial persistence at the epithelium, mechanistically driven by EspF-dependent depletion of icGCs. This loss disrupted the intercrypt mucus substructure, destabilizing the lattice that normally supports crypt plume mucus. As a result, plumes appeared sparse and unstable, creating permissive niches that followed outward dissemination of the pathogen into the FML. This model suggests that IML disruption reflects bacterial shedding toward other colonic regions or potential transmission to new hosts, rather than direct epithelial invasion. I wish to build on these findings by infecting mice with *C. rodentium* lacking EspF to directly visualize the intercrypt mucus substructure and determine whether the improved GC survival observed during such infections is reflected in better maintenance of intercrypt mucus. Additionally, infecting Muc2-deficient mice with EspF mutants will help determine

whether differences in infection and shedding between WT and KO hosts depend on the presence of the inner mucus layer. It will be important to define the molecular pathways downstream of EspF that mediate transcriptional reprogramming and ER stress in GCs, and to test whether host-directed interventions can preserve icGC survival and intercrypt mucus integrity during infection.

In **Paper III**, we showed that senGCs are important sentinel defenders of crypt entrances. When pathogens cross the IML and reach the crypt openings (as demonstrated in Paper II), senGCs become activated and help maintain crypt sterility through rapid inducible mucus secretion, by sustaining baseline mucus output under infection, and by possibly even driving reprogramming of crypt GCs to mount protective responses against crypt infiltration. Consistent with this role, senGC-deficient mice exhibited a tendency toward higher mucus penetrability, which may in turn predispose to faster IML disruption, underscoring the contribution of senGCs to maintaining barrier stability at early stages of pathogen challenge.

Building on the results that senGCs expand as *Nlrp6*$^{+}$ GCs with a previously unreported potential for deep crypt secretion during infection, it would be interesting to map GC reprogramming at single-cell resolution by profiling *Nlrp6*$^{fl/fl}$ and *Nlrp6*$^{\Delta GC}$ epithelium over the infection course. This could define how the GC landscape is remodeled (GC subset frequencies, transcriptional states) and identify mediators that induce this plasticity including microbial metabolites, cytokines (e.g., IL-18), and epithelial stress pathways. In parallel, these results suggest a developmental angle worth testing: the neonatal shift from senGC-independent secretion to senGC-dependent mucus release observed in **Paper I**, where deep crypt secretion accompanies early microbial exposure. A key question is whether neonatal deep crypt secretors are Nlrp6^{+}, and whether their program resembles that of infection-induced deep crypt *Nlrp6*$^{+}$ GCs. If so, the “stabilizing IML, high-exposure” state of early life may parallel the “disrupted IML, pathogen-inundated” state during infection. Addressing this could unify the concept of crypt defense across development and disease, positioning Nlrp6-centered GC plasticity as a common mediator in both scenarios.

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Generative AI was used to improve the grammar and readability of certain portions of this text. These suggestions were reviewed and subsequently improved upon by the author.

References

1. Johansson, M.E., J.M. Larsson, and G.C. Hansson, *The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions.* Proc Natl Acad Sci U S A, 2011. **108 Suppl 1**(Suppl 1): p. 4659-65.
2. Witten, J., T. Samad, and K. Ribbeck, *Selective permeability of mucus barriers.* Curr Opin Biotechnol, 2018. **52**: p. 124-133.
3. Ermund, A., et al., *Studies of mucus in mouse stomach, small intestine, and colon. I. Gastrointestinal mucus layers have different properties depending on location as well as over the Peyer's patches.* Am J Physiol Gastrointest Liver Physiol, 2013. **305**(5): p. G341-7.
4. Hansson, G.C., *Mucins and the Microbiome.* Annu Rev Biochem, 2020. **89**: p. 769-793.
5. Nyström, E.E.L., et al., *Calcium-activated Chloride Channel Regulator 1 (CLCA1) Controls Mucus Expansion in Colon by Proteolytic Activity.* EBioMedicine, 2018. **33**: p. 134-143.
6. van der Post, S., et al., *Structural weakening of the colonic mucus barrier is an early event in ulcerative colitis pathogenesis.* Gut, 2019. **68**(12): p. 2142-2151.
7. Jabbar, K.S., et al., *Association between Brachyspira and irritable bowel syndrome with diarrhoea.* Gut, 2021. **70**(6): p. 1117-1129.
8. Javitt, G., et al., *Intestinal Gel-Forming Mucins Polymerize by Disulfide-Mediated Dimerization of D3 Domains.* J Mol Biol, 2019. **431**(19): p. 3740-3752.
9. Birchenough, G.M.H., et al., *New developments in goblet cell mucus secretion and function.* Mucosal Immunology, 2015. **8**(4): p. 712-719.
10. Ambort, D., et al., *Calcium and pH-dependent packing and release of the gel-forming MUC2 mucin.* Proc Natl Acad Sci U S A, 2012. **109**(15): p. 5645-50.
11. Recktenwald, C.V. and G.C. Hansson, *The Reduction-insensitive Bonds of the MUC2 Mucin Are Isopeptide Bonds.* J Biol Chem, 2016. **291**(26): p. 13580-90.
12. Sharpen, J.D.A., et al., *Transglutaminase 3 crosslinks the secreted gel-forming mucus component Mucin-2 and stabilizes the colonic mucus layer.* Nat Commun, 2022. **13**(1): p. 45.
13. Schroeder, B.O., et al., *Obesity-associated microbiota contributes to mucus layer defects in genetically obese mice.* J Biol Chem, 2020. **295**(46): p. 15712-15726.

14. Macierzanka, A., A.R. Mackie, and L. Krupa, *Permeability of the small intestinal mucus for physiologically relevant studies: Impact of mucus location and ex vivo treatment.* Sci Rep, 2019. **9**(1): p. 17516.
15. Petersson, J., et al., *Importance and regulation of the colonic mucus barrier in a mouse model of colitis.* Am J Physiol Gastrointest Liver Physiol, 2011. **300**(2): p. G327-33.
16. Johansson, M.E., et al., *Normalization of Host Intestinal Mucus Layers Requires Long-Term Microbial Colonization.* Cell Host Microbe, 2015. **18**(5): p. 582-92.
17. Gustafsson, J.K., et al., *An ex vivo method for studying mucus formation, properties, and thickness in human colonic biopsies and mouse small and large intestinal explants.* Am J Physiol Gastrointest Liver Physiol, 2012. **302**(4): p. G430-8.
18. Johansson, M.E., et al., *The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria.* Proc Natl Acad Sci U S A, 2008. **105**(39): p. 15064-9.
19. Bergstrom, K., et al., *Proximal colon-derived O-glycosylated mucus encapsulates and modulates the microbiota.* Science, 2020. **370**(6515): p. 467-472.
20. Hugenholtz, F. and W.M. de Vos, *Mouse models for human intestinal microbiota research: a critical evaluation.* Cell Mol Life Sci, 2018. **75**(1): p. 149-160.
21. Nyström, E.E.L., et al., *An intercrypt subpopulation of goblet cells is essential for colonic mucus barrier function.* Science, 2021. **372**(6539).
22. Luis, A.S., et al., *A single sulfatase is required to access colonic mucin by a gut bacterium.* Nature, 2021. **598**(7880): p. 332-337.
23. Haber, A.L., et al., *A single-cell survey of the small intestinal epithelium.* Nature, 2017. **551**(7680): p. 333-339.
24. Wang, Y., et al., *Single-cell transcriptome analysis reveals differential nutrient absorption functions in human intestine.* J Exp Med, 2020. **217**(2).
25. Parikh, K., et al., *Colonic epithelial cell diversity in health and inflammatory bowel disease.* Nature, 2019. **567**(7746): p. 49-55.
26. Ehrencrona, E., et al., *The IgGFc-binding protein FCGBP is secreted with all GDPH sequences cleaved but maintained by interfragment disulfide bonds.* J Biol Chem, 2021. **297**(1): p. 100871.
27. Lo, Y.H., et al., *Transcriptional Regulation by ATOH1 and its Target SPDEF in the Intestine.* Cell Mol Gastroenterol Hepatol, 2017. **3**(1): p. 51-71.
28. Gregorieff, A., et al., *The ets-domain transcription factor Spdef promotes maturation of goblet and paneth cells in the intestinal epithelium.* Gastroenterology, 2009. **137**(4): p. 1333-45.e1-3.

29. Noah, T.K., et al., *SAM pointed domain ETS factor (SPDEF) regulates terminal differentiation and maturation of intestinal goblet cells.* Exp Cell Res, 2010. **316**(3): p. 452-65.
30. van der Flier, L.G. and H. Clevers, *Stem cells, self-renewal, and differentiation in the intestinal epithelium.* Annu Rev Physiol, 2009. **71**: p. 241-60.
31. *Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris.* Nature, 2018. **562**(7727): p. 367-372.
32. Kulkarni, D.H., et al., *Goblet cell associated antigen passages support the induction and maintenance of oral tolerance.* Mucosal Immunol, 2020. **13**(2): p. 271-282.
33. Noah, T.K., B. Donahue, and N.F. Shroyer, *Intestinal development and differentiation.* Exp Cell Res, 2011. **317**(19): p. 2702-10.
34. Koo, B.K., et al., *Porcupine inhibitor suppresses paracrine Wnt-driven growth of Rnf43;Znrf3-mutant neoplasia.* Proc Natl Acad Sci U S A, 2015. **112**(24): p. 7548-50.
35. Birchenough, G.M., et al., *A sentinel goblet cell guards the colonic crypt by triggering Nlrp6-dependent Muc2 secretion.* Science, 2016. **352**(6293): p. 1535-42.
36. Johansson, M.E., *Fast renewal of the distal colonic mucus layers by the surface goblet cells as measured by in vivo labeling of mucin glycoproteins.* PLoS One, 2012. **7**(7): p. e41009.
37. Javitt, G., et al., *Assembly Mechanism of Mucin and von Willebrand Factor Polymers.* Cell, 2020. **183**(3): p. 717-729.e16.
38. Tsuru, A., et al., *Negative feedback by IRE1β optimizes mucin production in goblet cells.* Proc Natl Acad Sci U S A, 2013. **110**(8): p. 2864-9.
39. Park, S.W., et al., *The protein disulfide isomerase AGR2 is essential for production of intestinal mucus.* Proc Natl Acad Sci U S A, 2009. **106**(17): p. 6950-5.
40. Zhao, F., et al., *Disruption of Paneth and goblet cell homeostasis and increased endoplasmic reticulum stress in Agr2-/- mice.* Dev Biol, 2010. **338**(2): p. 270-9.
41. Jaramillo, A.M., et al., *Different Munc18 proteins mediate baseline and stimulated airway mucin secretion.* JCI Insight, 2019. **4**(6).
42. Cornick, S., et al., *VAMP8-mediated MUC2 mucin exocytosis from colonic goblet cells maintains innate intestinal homeostasis.* Nat Commun, 2019. **10**(1): p. 4306.
43. Neutra, M.R., L.J. O'Malley, and R.D. Specian, *Regulation of intestinal goblet cell secretion. II. A survey of potential secretagogues.* Am J Physiol, 1982. **242**(4): p. G380-7.

44. Specian, R.D. and M.R. Neutra, *Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine.* J Cell Biol, 1980. **85**(3): p. 626-40.
45. Phillips, T.E., *Both crypt and villus intestinal goblet cells secrete mucin in response to cholinergic stimulation.* Am J Physiol, 1992. **262**(2 Pt 1): p. G327-31.
46. Halm, D.R. and S.T. Halm, *Secretagogue response of goblet cells and columnar cells in human colonic crypts.* Am J Physiol, 1999. **277**(3): p. C501-22.
47. Yang, D., et al., *Nociceptor neurons direct goblet cells via a CGRP-RAMP1 axis to drive mucus production and gut barrier protection.* Cell, 2022. **185**(22): p. 4190-4205.e25.
48. Sovran, B., et al., *Age-associated Impairment of the Mucus Barrier Function is Associated with Profound Changes in Microbiota and Immunity.* Sci Rep, 2019. **9**(1): p. 1437.
49. Grootjans, J., I.H. Hundscheid, and W.A. Buurman, *Goblet cell compound exocytosis in the defense against bacterial invasion in the colon exposed to ischemia-reperfusion.* Gut Microbes, 2013. **4**(3): p. 232-5.
50. Johansson, M.E., et al., *Bacteria penetrate the inner mucus layer before inflammation in the dextran sulfate colitis model.* PLoS One, 2010. **5**(8): p. e12238.
51. Hansen, A.K., et al., *Impact of the gut microbiota on rodent models of human disease.* World J Gastroenterol, 2014. **20**(47): p. 17727-36.
52. Johansson, M.E., et al., *Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis.* Gut, 2014. **63**(2): p. 281-91.
53. Van der Sluis, M., et al., *Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection.* Gastroenterology, 2006. **131**(1): p. 117-29.
54. Gersemann, M., et al., *Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis.* Differentiation, 2009. **77**(1): p. 84-94.
55. Liu, J.Z. and C.A. Anderson, *Genetic studies of Crohn's disease: past, present and future.* Best Pract Res Clin Gastroenterol, 2014. **28**(3): p. 373-86.
56. Adolph, T.E., et al., *Paneth cells as a site of origin for intestinal inflammation.* Nature, 2013. **503**(7475): p. 272-6.
57. Larsson, J.M., et al., *Altered O-glycosylation profile of MUC2 mucin occurs in active ulcerative colitis and is associated with increased inflammation.* Inflamm Bowel Dis, 2011. **17**(11): p. 2299-307.
58. Wang, R., et al., *Gut microbiota shape the inflammatory response in mice with an epithelial defect.* Gut Microbes, 2021. **13**(1): p. 1-18.

59. Bergström, J.H., et al., *Gram-positive bacteria are held at a distance in the colon mucus by the lectin-like protein ZG16.* Proc Natl Acad Sci U S A, 2016. **113**(48): p. 13833-13838.
60. Haider, K., et al., *Production of mucinase and neuraminidase and binding of Shigella to intestinal mucin.* J Diarrhoeal Dis Res, 1993. **11**(2): p. 88-92.
61. van der Post, S., et al., *Site-specific O-glycosylation on the MUC2 mucin protein inhibits cleavage by the Porphyromonas gingivalis secreted cysteine protease (RgpB).* J Biol Chem, 2013. **288**(20): p. 14636-14646.
62. Luo, Q., et al., *Enterotoxigenic Escherichia coli secretes a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells.* Infect Immun, 2014. **82**(2): p. 509-21.
63. Sansonetti, P.J. and A. Phalipon, *M cells as ports of entry for enteroinvasive pathogens: mechanisms of interaction, consequences for the disease process.* Semin Immunol, 1999. **11**(3): p. 193-203.
64. Clark, M.A., et al., *Preferential interaction of Salmonella typhimurium with mouse Peyer's patch M cells.* Res Microbiol, 1994. **145**(7): p. 543-52.
65. Fasciano, A.C., et al., *Yersinia pseudotuberculosis YopE prevents uptake by M cells and instigates M cell extrusion in human ileal enteroid-derived monolayers.* Gut Microbes, 2021. **13**(1): p. 1988390.
66. Bergstrom, K.S., et al., *Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa.* PLoS Pathog, 2010. **6**(5): p. e1000902.
67. Schauer, D.B. and S. Falkow, *Attaching and effacing locus of a Citrobacter freundii biotype that causes transmissible murine colonic hyperplasia.* Infect Immun, 1993. **61**(6): p. 2486-92.
68. Schauer, D.B. and S. Falkow, *The eae gene of Citrobacter freundii biotype 4280 is necessary for colonization in transmissible murine colonic hyperplasia.* Infect Immun, 1993. **61**(11): p. 4654-61.
69. Willing, B.P., et al., *Altering host resistance to infections through microbial transplantation.* PLoS One, 2011. **6**(10): p. e26988.
70. Ghosh, S., et al., *Colonic microbiota alters host susceptibility to infectious colitis by modulating inflammation, redox status, and ion transporter gene expression.* Am J Physiol Gastrointest Liver Physiol, 2011. **301**(1): p. G39-49.
71. Crepin, V.F., et al., *Citrobacter rodentium mouse model of bacterial infection.* Nat Protoc, 2016. **11**(10): p. 1851-76.
72. Wiles, S., et al., *Organ specificity, colonization and clearance dynamics in vivo following oral challenges with the murine pathogen Citrobacter rodentium.* Cell Microbiol, 2004. **6**(10): p. 963-72.

73. Hopkins, E.G.D., et al., *Intestinal Epithelial Cells and the Microbiome Undergo Swift Reprogramming at the Inception of Colonic Citrobacter rodentium Infection.* mBio, 2019. **10**(2).
74. Garmendia, J., G. Frankel, and V.F. Crepin, *Enteropathogenic and enterohemorrhagic Escherichia coli infections: translocation, translocation, translocation.* Infect Immun, 2005. **73**(5): p. 2573-85.
75. Nakanishi, N., et al., *Regulation of virulence by butyrate sensing in enterohaemorrhagic Escherichia coli.* Microbiology (Reading), 2009. **155**(Pt 2): p. 521-530.
76. Umanski, T., I. Rosenshine, and D. Friedberg, *Thermoregulated expression of virulence genes in enteropathogenic Escherichia coli.* Microbiology (Reading), 2002. **148**(Pt 9): p. 2735-2744.
77. Reading, N.C., et al., *The two-component system QseEF and the membrane protein QseG link adrenergic and stress sensing to bacterial pathogenesis.* Proc Natl Acad Sci U S A, 2009. **106**(14): p. 5889-94.
78. Lopez, C.A., et al., *Virulence factors enhance Citrobacter rodentium expansion through aerobic respiration.* Science, 2016. **353**(6305): p. 1249-53.
79. Barthold, S.W., et al., *The etiology of transmissible murine colonic hyperplasia.* Lab Anim Sci, 1976. **26**(6 Pt 1): p. 889-94.
80. Berger, C.N., et al., *Citrobacter rodentium Subverts ATP Flux and Cholesterol Homeostasis in Intestinal Epithelial Cells In Vivo.* Cell Metab, 2017. **26**(5): p. 738-752.e6.
81. Kim, Y.G., et al., *The Nod2 sensor promotes intestinal pathogen eradication via the chemokine CCL2-dependent recruitment of inflammatory monocytes.* Immunity, 2011. **34**(5): p. 769-80.
82. Li, Y., et al., *TLR9 Regulates the NF-κB-NLRP3-IL-1β Pathway Negatively in Salmonella-Induced NKG2D-Mediated Intestinal Inflammation.* J Immunol, 2017. **199**(2): p. 761-773.
83. Friedrich, C., et al., *MyD88 signaling in dendritic cells and the intestinal epithelium controls immunity against intestinal infection with C. rodentium.* PLoS Pathog, 2017. **13**(5): p. e1006357.
84. Kayagaki, N., et al., *Non-canonical inflammasome activation targets caspase-11.* Nature, 2011. **479**(7371): p. 117-21.
85. Kayagaki, N., et al., *Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling.* Nature, 2015. **526**(7575): p. 666-71.
86. Shi, J., et al., *Inflammatory caspases are innate immune receptors for intracellular LPS.* Nature, 2014. **514**(7521): p. 187-92.
87. Liu, Z., et al., *Role of inflammasomes in host defense against Citrobacter rodentium infection.* J Biol Chem, 2012. **287**(20): p. 16955-64.

88. Pallett, M.A., et al., *Bacterial virulence factor inhibits caspase-4/11 activation in intestinal epithelial cells.* Mucosal Immunol, 2017. **10**(3): p. 602-612.
89. Seo, S.U., et al., *Intestinal macrophages arising from CCR2(+) monocytes control pathogen infection by activating innate lymphoid cells.* Nat Commun, 2015. **6**: p. 8010.
90. Sakamoto, K., et al., *IL-22 Controls Iron-Dependent Nutritional Immunity Against Systemic Bacterial Infections.* Sci Immunol, 2017. **2**(8).
91. Hood, M.I. and E.P. Skaar, *Nutritional immunity: transition metals at the pathogen-host interface.* Nat Rev Microbiol, 2012. **10**(8): p. 525-37.
92. Waldschmitt, N., et al., *The regenerating family member 3 β instigates IL-17A-mediated neutrophil recruitment downstream of NOD1/2 signalling for controlling colonisation resistance independently of microbiota community structure.* Gut, 2019. **68**(7): p. 1190-1199.
93. Xiao, Y., et al., *Interleukin-33 Promotes REG3γ Expression in Intestinal Epithelial Cells and Regulates Gut Microbiota.* Cell Mol Gastroenterol Hepatol, 2019. **8**(1): p. 21-36.
94. Higgins, L.M., et al., *Citrobacter rodentium infection in mice elicits a mucosal Th1 cytokine response and lesions similar to those in murine inflammatory bowel disease.* Infect Immun, 1999. **67**(6): p. 3031-9.
95. Atarashi, K., et al., *Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells.* Cell, 2015. **163**(2): p. 367-80.
96. Ahlfors, H., et al., *IL-22 fate reporter reveals origin and control of IL-22 production in homeostasis and infection.* J Immunol, 2014. **193**(9): p. 4602-13.
97. Chassaing, B., et al., *Dextran sulfate sodium (DSS)-induced colitis in mice.* Curr Protoc Immunol, 2014. **104**: p. 15.25.1-15.25.14.
98. Atuma, C., et al., *The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo.* Am J Physiol Gastrointest Liver Physiol, 2001. **280**(5): p. G922-9.
99. Wlodarska, M., et al., *NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion.* Cell, 2014. **156**(5): p. 1045-59.
100. Volk, J.K., et al., *The Nlrp6 inflammasome is not required for baseline colonic inner mucus layer formation or function.* J Exp Med, 2019. **216**(11): p. 2602-2618.
101. Nyström, E.E.L., et al., *Calcium-activated chloride channel regulator 1 (CLCA1) forms non-covalent oligomers in colonic mucus and has mucin 2-processing properties.* J Biol Chem, 2019. **294**(45): p. 17075-17089.

102. Leverkoehne, I. and A.D. Gruber, *The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells.* J Histochem Cytochem, 2002. **50**(6): p. 829-38.
103. Lorand, L. and R.M. Graham, *Transglutaminases: crosslinking enzymes with pleiotropic functions.* Nat Rev Mol Cell Biol, 2003. **4**(2): p. 140-56.
104. Hansson, G.C. and M.E. Johansson, *The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria.* Gut Microbes, 2010. **1**(1): p. 51-54.
105. Gustafsson, J.K., et al., *Dynamic changes in mucus thickness and ion secretion during Citrobacter rodentium infection and clearance.* PLoS One, 2013. **8**(12): p. e84430.
106. Das, S., et al., *Neutrophils and galectin-3 defend mice from lethal bacterial infection and humans from acute respiratory failure.* Nature Communications, 2024. **15**(1): p. 4724.
107. Bülck, C., et al., *Proteolytic processing of galectin-3 by meprin metalloproteases is crucial for host-microbiome homeostasis.* Sci Adv, 2023. **9**(13): p. eadf4055.
108. Burgueño, J.F., et al., *Epithelial TLR4 Signaling Activates DUOX2 to Induce Microbiota-Driven Tumorigenesis.* Gastroenterology, 2021. **160**(3): p. 797-808.e6.
109. Sommer, F. and F. Bäckhed, *The gut microbiota engages different signaling pathways to induce Duox2 expression in the ileum and colon epithelium.* Mucosal Immunol, 2015. **8**(2): p. 372-9.
110. Gustafsson, J.K., et al., *Intestinal goblet cells sample and deliver lumenal antigens by regulated endocytic uptake and transcytosis.* Elife, 2021. **10**.
111. Xia, X., et al., *EspF is crucial for Citrobacter rodentium-induced tight junction disruption and lethality in immunocompromised animals.* PLoS Pathog, 2019. **15**(6): p. e1007898.