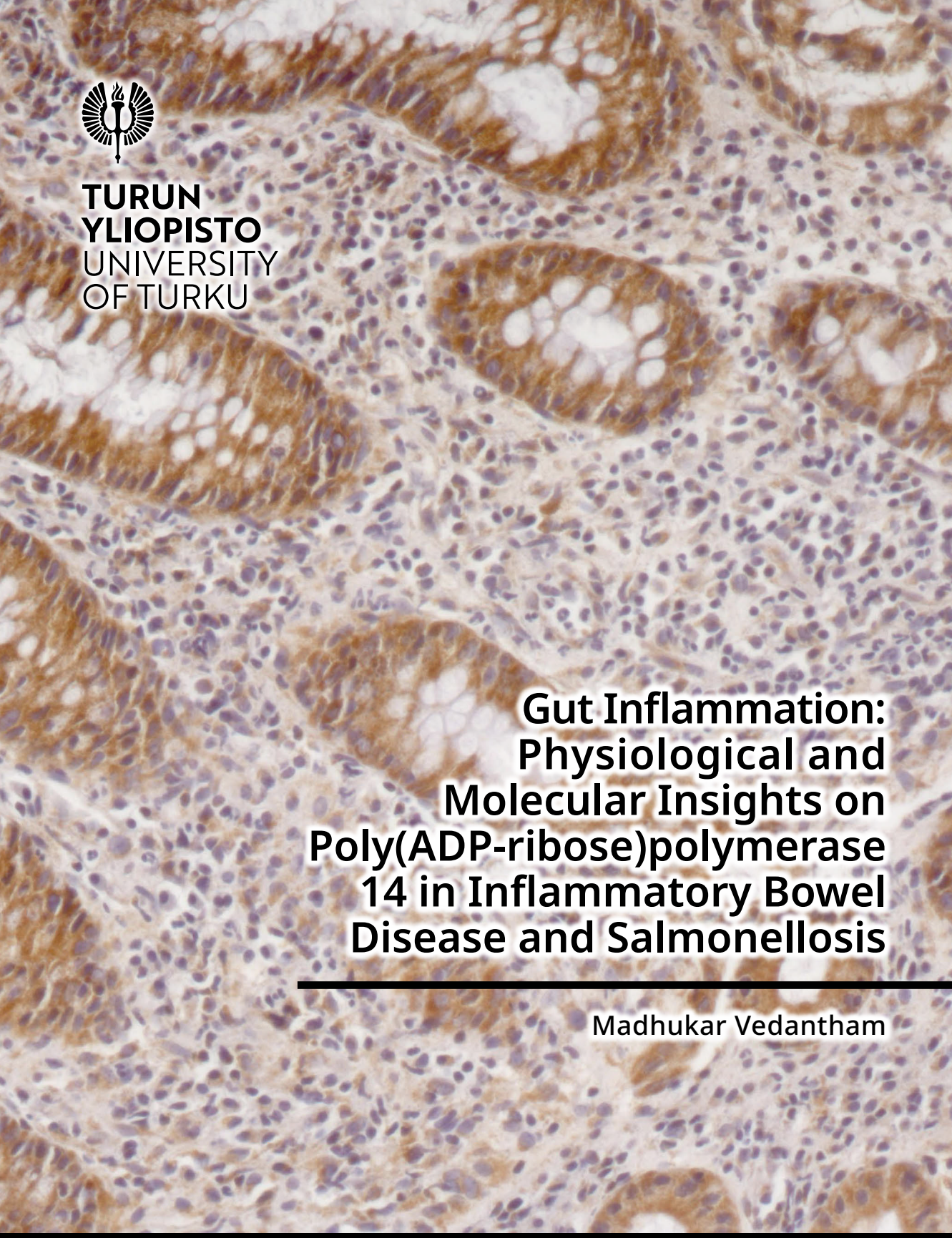

TURUN YLIOPISTO
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Gut Inflammation: Physiological and Molecular Insights on Poly(ADP-ribose)polymerase 14 in Inflammatory Bowel Disease and Salmonellosis

Madhukar Vedantham

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To my family and friends

UNIVERSITY OF TURKU
Faculty of Medicine
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MADHUKAR VEDANTHAM: Gut Inflammation: Physiological and Molecular Insights on Poly(ADP-ribose) polymerase 14 in Inflammatory Bowel Disease and Salmonellosis
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ABSTRACT

Inflammatory bowel disease (IBD) and *Salmonella* infection (salmonellosis) are characterized by disruption of the intestinal barrier and severe gut inflammation. A multitude of cell signaling pathways regulate the gut immunological responses. Protein ADP-ribosylation and the enzymes catalyzing this modification, that is, poly(ADP-ribose) polymerases (PARPs), have gained attention in the past few years for their potential roles in the regulation of immunological responses. Yet, the mechanistic basis and more importantly the physiological relevance have largely remained elusive.

This study focuses on PARP14 in gut inflammation. PARP14 expression and function were examined using colon biopsies of IBD vs control patients and tissue material from two mouse models, that is, from the oral dextran sulfate sodium (DSS) exposure colitis model and the oral *Salmonella* Typhimurium infection model. PARP14 was predominantly expressed by the epithelial cells (with granular cytosolic staining) in human IBD and control patient colons. Analysis of bulk tissue transcriptomic data revealed higher PARP14 expression in the colon of IBD patients as compared to control patients. In mice, upon DSS-treatment and *Salmonella* infection, majority of the cells expressing PARP14 were epithelial cells, along with some mucosal macrophages. Analysis of *Salmonella* infection single-cell RNA-Seq data of the small intestine epithelium revealed pronounced PARP14 expression in the enterocytes and Tuft cells. Based on the qPCR analysis, PARP14 expression was higher in the colon of DSS-treated mice as compared to controls. Body-wide genetic PARP14 deficiency was associated with exacerbated colitis in both mouse models. Most importantly, the colon histopathology analysis revealed more severe epithelial barrier damage – including extensive epithelial erosion, Goblet cell loss, and increased immune cell infiltration – in the PARP14-deficient mice. PacBio sequencing (microbiota analysis), flow cytometry (leukocyte population analysis) and RNA-Seq (transcriptomic analysis) were used to study features that potentially associate with the pronounced pathologies of PARP14-deficient mice. While no significant microbiota and leukocyte population differences were detected between wt and PARP14-deficient mice, the transcriptomic analysis indicated a critical regulatory role for PARP14. Bulk RNA sequencing of colon samples showed dysregulated expression of inflammation- and infection-response genes in PARP14-

deficient mice both prior and after DSS exposure, while *Salmonella* infected knockouts lacked key gene expression signatures related to cell adhesion, cell proliferation, and cytoskeletal organization.

In conclusion, this study uncovered a previously unknown role of PARP14 in gut inflammation. PARP14 appears to play a protective role in the gut by maintaining epithelial integrity and modulating mucosal inflammation, possibly as a transcriptional regulator. Biomarker potential of PARP14 in gut disorders merits further investigation.

KEYWORDS: inflammatory bowel disease, Salmonella, colitis, inflammation, infection, PARP14

TURUN YLIOPISTO
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TIIVISTELMÄ

Tulehdukselliselle suolistosairaudelle (inflammatory bowel disease, IBD) ja *Salmonella* infektiolle (salmonelloosi) on tunnusomaista vaurioitunut suolisto-epiteelisolukko ja vakava suolistotulehdus. Lukuisat solujen signalointireitit säätelevät suoliston immunologisia vasteita. Proteiinien ADP-ribosylaatio ja tätä modifikaatiota katalysoivat entsyymit eli poly(ADP-riboosi)polymeraasit (PARP:t) ovat viime vuosina saaneet huomiota mahdollisista rooleistaan immunologisten vasteiden säätelyssä. Säätelyn molekyylimekanismit ja mikä tärkeintä sen fysiologiset merkitykset ovat suurelta osin jääneet vielä tuntemattomiksi.

Tämä tutkimus keskittyy PARP14 proteiiniin suolistotulehduksessa. PARP14 proteiinin ilmentymistä ja toimintaa tutkittiin käyttämällä IBD-potilaiden ja verrokkipotilaiden paksusuolen biopsioita ja kudosmateriaalia kahdesta hiirimallista, toisin sanoen dekstraanisulfaattinatrium (DSS) koliittimallista ja Salmonella infektiomallista. PARP14 proteiinia ilmensivät pääasiassa epiteelisolut (rakeisella sytosoliväräyksellä) IBD-potilaiden ja verrokkipotilaiden paksusuolessa. Kudosten transkriptominen analyysi paljasti korkeamman PARP14 ekspression IBD-potilaiden paksusuolessa verrattuna kontrollipotilaisiin. Hiirten epiteelisolut, samoin kuin limakalvomakrofagit, ilmensivät voimakkaasti PARP14 proteiinia DSS käsittelyn ja Salmonella infektion jälkeen. Salmonella infektion yksisolu-RNA-Seq-analyysi ohutsuolen epiteelisolukosta paljasti voimakkaan PARP14 ekspression enterosyyteissä ja Tuft-soluissa. PARP14 ilmentyminen oli korkeampi DSS käsiteltyjen hiirten paksusuolessa verrattuna kontrolleihin kvantitatiivisen PCR analyysin perusteella. PARP14 proteiinin puute aiheutti voimistuneen paksusuolen-tulehduksen molemmissa hiirimalleissa. Paksusuolen histopatologinen analyysi paljasti vakavampia epiteelisolukon vaurioita PARP14 poistogeenisillä hiirillä, mukaan lukien laajan epiteelisolueroosion, pikarisolujen häviämisen ja lisääntyneen immuunisolujen infiltraation. PacBio-sekvensointia (mikrobiota-analyysi), virtaussytometriaa (leukosyyttipopulaatioanalyysi) ja RNA-Seq-menetelmää (transkriptominen analyysi) käytettiin sellaisten piirteiden tutkimiseen, jotka mahdollisesti liittyvät PARP14 poistogeenisten hiirten voimistuneisiin patologioihin. Merkittäviä mikrobiota- ja leukosyyttipopulaatioeroja ei havaittu villityypin ja PARP14 poistogeenisten hiirten välillä, mutta transkriptominen analyysi osoitti kriittisen säätelevän roolin PARP14 proteiinille. Paksusuolinäytteiden RNA-sekvensointi

osoitti tulehdus- ja infektiovastegeenien säätelemätöntä ilmentymistä PARP14 poistogeenisissä hiirissä sekä ennen DSS käsittelyä että sen jälkeen. Salmonella infektion saaneista PARP14 poistogeenisistä hiiristä puolestaan paljastui ongelmia siinä miten solujen adheesioon, solujen lisääntymiseen ja sytoskeletaaliseen organisaatioon liittyviä geenejä ilmennettiin.

Yhteenvetona voidaan todeta, että tämä tutkimus paljasti aiemmin tuntemattoman roolin PARP14 proteiinille suolistotulehduksessa. PARP14 proteiinilla näyttäisi olevan suojaava rooli suolistossa ylläpitämällä epiteelisolukon eheyttä ja muokkaamalla limakalvon tulehdusreaktiota, mahdollisesti transkriptionaalisena säätelijänä. PARP14 proteiinin potentiaali suolistosairauksien biomarkkerina ansaitsee lisätutkimuksia.

AVAINSANAT: tulehduksellinen suolistosairaus, *Salmonella*, koliitti, tulehdus, infektio, PARP14

Table of Contents

Abbreviations

5-ASA	5-aminosalicylic acid
ADP	Adenosine diphosphate
AhR	aryl hydrocarbon receptor
AMP	Antimicrobial peptides
ART	ADP-ribosyltransferase
ARTCs	cholera toxin-like ARTs
ARTDs	diphtheria toxin-like ARTs
BP	biological process
CCL	chemokine (C-C motif) ligand
CD	Crohn's disease
CDC	Centre for Disease Control and Prevention
CFU	colony forming units
DAB	3,3'-Diaminobenzidine
DC	Dendritic cells
DEG	differentially expressed gene
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
FBS	Fetal bovine serum
FCS	fetal calf serum
FFPE	formalin-fixed paraffin-embedded
FPKM	fragments per kilobase per million mapped fragments
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GO	Gene ontology
H&E	hematoxylin and eosin
HBSS	Hank's balanced salt solution
HDAC	histone deacetylase
HRP	horse radish peroxidase
H-Y-E	Histidine-Tyrosine-Glutamic acid
H-Y-L	Histidine-Tyrosine-Leucine

IBD	Inflammatory Bowel Disease
ICAM-1	intercellular adhesion molecule-1
IEC	Intestinal epithelial cells
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate lymphoid cells
iNTS	invasive non-typhoidal *Salmonella*
KEGG	Kyoto Encyclopedia of Genes and Genomes
KH domain	K-homology domain
KO mice	knockout mice
LB	Luria-Bertani
LP	lamina propria
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MLN	Mesenteric lymph nodes
mRNA	messenger RNA
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NLR	NOD-like receptor
OD	optical density
OTU	operational taxonomic unit
PAMPs	pathogen-associated molecular patterns
PARG	poly (ADP-ribose) glycohydrolase
PARP	poly (ADP-ribose) polymerase
PARPi	PARP inhibitors
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
PTM	post-translational modification
qPCR	quantitative PCR
RELM	resistin-like molecules
RNA	Ribonucleic acid
RRM	RNA recognition motif
rRNA	Ribosomal Ribonucleic acid
SCV	*Salmonella* containing vacuole
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPI	Salmonella pathogenicity island

STAT	signal transducer and activator of transcription
STm	*Salmonella enterica* serovar Typhimurium
T3SS	Type-3 secretory system
TFF	trefoil factor peptides
Tfh cells	T follicular helper cells
TGF-β	Transforming growth factor beta
Th cells	T helper cells
TLR	toll-like receptor
TNF	Tumor necrosis factor
Treg cells	T regulatory cells
UC	Ulcerative colitis
WHO	World Health Organization
WT mice	wild-type mice
WWE	Tryptophan-Tryptophan-Glutamic acid
YLD	years lived with disability

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Vedantham M**, Polari L, Poosakkannu A, Pinto RT, Sakari M, Laine J, Sipilä P, Määttä J, Gerke H, Rissanen T, Rantakari P, Toivola DM, Pulliainen AT. Body-wide genetic deficiency of poly(ADP-ribose) polymerase 14 sensitizes mice to colitis. *The FASEB Journal*. 2024; 38:e23775.

II **Vedantham M**, Polari L, Rissanen T, Pulliainen AT. Exacerbated salmonellosis in poly(ADP-ribose) polymerase 14 deficient mice. *Manuscript*

1 Introduction

Gastrointestinal inflammation manifests in a spectrum of disorders that impose a considerable burden on global health. Chronic conditions such as inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease, alongside acute infections like salmonellosis, offer distinct yet complementary models for studying intestinal inflammation. While these conditions differ in etiology and clinical presentation, both are characterized by complex immune responses and disruptions in normal tissue homeostasis.

ADP-ribosylation is a reversible post-translational modification that modulates a wide array of cellular functions including DNA repair, transcription, and signal transduction. Within this regulatory framework, PARP14 emerges as a protein of interest. As a member of the poly(ADP-ribose) polymerase (PARP) family, PARP14 functions primarily as a mono-ADP-ribosyltransferase. It possesses a multifaceted domain structure that includes RNA recognition motifs, macrodomains, and a catalytic region, suggesting a capacity to integrate diverse signaling cues.

Originally identified in the context of B cell function and Th2 cytokine signaling, PARP14 has since been implicated in the modulation of immune responses, particularly through its responsiveness to interferons and other inflammatory stimuli. Despite these insights, the role of PARP14 in the setting of gastrointestinal inflammation remains largely unexplored. In contrast to the extensive studies on other members of the PARP family, the specific expression patterns, regulatory mechanisms, and functional contributions of PARP14 in the inflamed gut have yet to be defined.

The focus of my PhD thesis is to establish a comprehensive understanding of PARP14 in the context of gastrointestinal inflammation. By examining both human colonic tissue and established murine models, this study seeks to elucidate the regulation and potential signaling roles of PARP14 in intestinal inflammation. This foundational insight is essential for advancing our understanding of the cellular processes governing inflammatory responses in the gut.

2 Review of the Literature

2.1 Gut and its immune system

Gastrointestinal (GI) tract is an active and complex system not only for digestion but also for the absorption of nutrients and general well-being. It is a chain of connected organs—the mouth, esophagus, stomach, small intestine, and large intestine—each carrying out a different function for the breakdown of food and absorption of nutrients. Other than its role in digestion, the gut is also a key defense against infection and contains an immeasurable amount of microorganisms, the combined total being called the gut microbiota. The gut microbiota is very well networked with the immune system, influencing inflammation and metabolic processes and playing a major role in the determination of homeostasis (Zmora et al., 2019). The intricacy of the anatomy and the multiplicity of functions of the gastrointestinal tract are merely an expression of its pivotal function in both physiological regulation and the etiopathogenesis of most diseases.

In Figure 1, the schematic comparison of the human and mouse GI tracts highlights both the shared and unique aspects of their organization. The human and mouse GI tract, while sharing a similar overall layout from the esophagus to the colon, there are notable differences that are critical when employing mouse models to study human gut diseases. For instance, cecum in mice is significantly larger compared to humans. It possesses a primary role of microbial fermentation and immune modulation in mice. This variation of the cecal structure can be responsible for determining the composition of gut microbiota as well as nature of immune response, factors that are essential when establishing the experimental results with respect to human disease. Additionally, variations in the structure of intestinal villus and crypt, as well as variation in distribution and organization of gut-associated lymphoid tissue, again illustrate species-specific features to be considered within translational studies (Hugenholtz & de Vos, 2018).

This PhD thesis leverages mouse models to investigate gastrointestinal infection and inflammation. In a healthy state, the gut maintains a finely tuned homeostasis that supports efficient digestion, absorption, and a balanced microbial community. However, when this equilibrium is disturbed by factors such as pathogens, dietary changes, or genetic predispositions, the result can be a spectrum of gastrointestinal

diseases marked by altered barrier function and dysregulated immune responses. In this context, the gut's immune system emerges as a central mediator, it not only detects and responds to these disturbances but also works to restore balance and repair tissue damage (Mowat & Agace, 2014). The ensuing literature review will examine these interconnected aspects in detail, thereby laying the groundwork for understanding the immune mechanisms that underpin gastrointestinal health and disease.

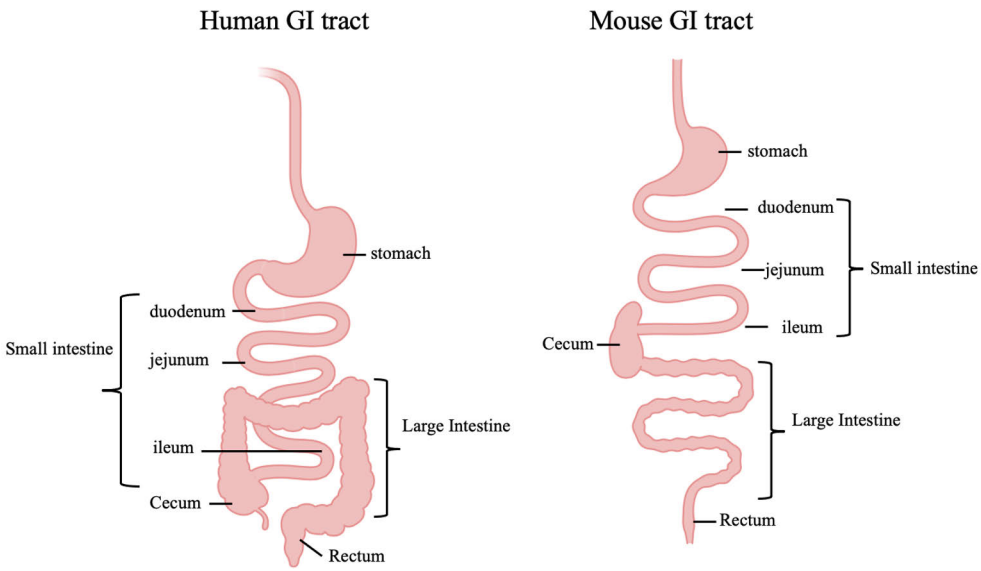


Figure 1. Simple illustration of human and mouse gastrointestinal tract. This illustration shows their shared and unique organization. Figure created with BioRender.com

2.1.1 Immune cells in the gut

The gastrointestinal tract harbors the body's largest collection of immune cells, constituting the gut-associated lymphoid tissue (GALT) and a broad network of immune populations in the mucosa. The goal of the gut immune system is two-fold: tolerate beneficial microbes and food antigens while mounting rapid defenses against pathogens. To achieve this, the GALT integrates elements of innate and adaptive immunity in the intestinal mucosa. Key components include:

- Innate immune cells: The intestinal lamina propria contains abundant resident macrophages and dendritic cells (DCs). These mononuclear phagocytes continuously sample the intestinal lumen in mice (sometimes extending dendrites between epithelial cells) and monitor for invading microbes (Niess et al., 2005; Rescigno et al., 2001). In the absence of infection or injury, they mediate tolerance,

for example, by causing the differentiation of regulatory T cells or secretion of IL-10. However, following detection of pathogen, macrophages and DCs induce activation of the immune system by secreting inflammatory cytokines (such as IL-1, IL-6, TNF) and presenting antigens to T cells (Isidro & Appleyard, 2016; Ma et al., 2019). Innate lymphoid cells (ILCs) are an additional important innate population; in the gut, group 3 ILCs (ILC3) respond to microbial signals by producing the cytokines IL-22 and IL-17 that help strengthen the epithelial barrier and recruit neutrophils (Peterson & Artis, 2014). Neutrophils are generally rare in healthy intestine but can rapidly infiltrate gut tissue when infection or injury is occurring, as occurs in both inflammatory bowel disease (IBD) flares and in response to *Salmonella* infection (Fournier & Parkos, 2012). Mast cells and eosinophils are also present in the intestinal mucosa (mostly in the colon) and ultimately contribute to local immune responses against helminths and in allergic responses (Ribatti, 2016; Walker et al., 2011).

- Adaptive immune cells: The gut mucosa is populated by a myriad of lymphocytes. T cells exist in organized structures (Peyer's patches and mesenteric lymph nodes) as well as dispersed in the lamina propria or the intraepithelial compartment (Ma et al., 2019). CD4 T helper cells in the lamina propria can differentiate into various functional subsets: Th1, Th2, Treg, Th17, Tfh and Th9. As an example, regulatory T cells (Tregs) produce IL-10 to dampen inflammation or maintain tolerance, while Th17 cells (driven by microbiota signals) produce IL-17 and IL-22 to enhance epithelial defenses, and Th9 cells which were identified in 2014 to produce IL-9 that promotes ulcerative colitis (Gerlach et al., 2014). It is thought that in patients suffering from inflammatory bowel disease, the pathology arises due to a poorly regulated ratio of effector Th17/Th1 and Tregs (Gomez-Bris et al., 2023). Intraepithelial lymphocytes are frequently CD8 T cells or cytotoxic T cells (consisting of cells such as γδ T cells) and reside within the epithelium; they provide front-line surveillance against infections and can kill infected epithelial cells (Y. Chen et al., 2024). Many B cells in gut-associated lymphoid tissues class switch to produce IgA, which is the dominant isotype of antibody made in the gut. IgA-producing plasma cells exit the germinal centers in gut-associated lymphoid tissues and flood the lamina propria with IgA dimers that can transcytose into the gut lumen. Secretory IgA can bind microbes and/or toxins, allowing for neutralization and preventing colonization of epithelial cells in the gut; importantly, secretory IgA does not induce inflammation and helps maintain a mutualistic relationship with the commensals (Carreto-Binaghi et al., 2024; Siniscalco et al., 2024). In fact, mice without IgA or the critical cytokines (for example, IL-22 or IL-17) that govern IgA responses often harbor dysregulated microbial communities and increased susceptibility to gut infections (Agace & McCoy, 2017; Kabat et al., 2014).

- Gut-associated lymphoid structures: Peyer's patches (in the ileum) and dispersed lymphoid follicles (along the intestine) are inductive sites for the adaptive response (Fig. 2). M cells (microfold cells) in Peyer's patch epithelia present antigens to underlying DCs and B cell follicles. DCs migrate into mesenteric lymph nodes (Pabst & Bernhardt, 2013), and initiate adaptive immunity in order to keep the intestinal barrier intact (Dillon & Lo, 2019). Mesenteric lymph nodes drain the intestine and are important in inducing tolerance to dietary antigens and commensals; germ-free animals have underdeveloped GALT, showing how microbial exposure drives immune development (Round & Mazmanian, 2009).

In addition to these key components, the intestinal immune system comprises of cells that have dynamic immune functions: intestinal epithelial cells (IECs) (Goto, 2019), goblet cells (Knoop & Newberry, 2018), Paneth cells (Wallaeys et al., 2023), and M cells (Fig. 2). There is dynamic cross-communication in homeostasis between these cells, immune cells and the gut microbiota. Commensal bacteria and their metabolites (short-chain fatty acids, e.g.) direct differentiation of ILCs and of T cells towards a regulatory and IgA-biasing direction that constrains excessive inflammation and promotes mucosal tolerance. Conversely, composition of the microbiota is regulated by the immune system by IgA and by antimicrobial peptides (Caruso et al., 2020). Disequilibrium in this state can lead to disease: over-activation of the immune system leads to inflammation (e.g., in IBD), whereas under-activation can allow pathogenic infection. Hence, the immunology of the gut is a precisely tuned orchestra of cells that collectively keep intestinal health by a balance between defense and tolerance.

2.1.2 Epithelial barrier and its importance during inflammation

The intestinal epithelial barrier is the front line of defense of the gut against inflammation. It is a sheet of epithelial cells bound tightly together by junctional complexes, reinforced by secreted mucus and antimicrobial components. It is a physical and chemical barrier that keeps the trillions of luminal microbes away from the host's sterile internal environment (Lechuga et al., 2023). Specifically, in the colon, the epithelium is covered by a two-tier mucus system: one that is dense and well-adherent to the epithelium and bacterium-free under normal circumstances, and one that is loose and inhabited by commensal microbes (Johansson & Hansson, 2016) (Fig. 2). Its internal mucus layer, organized in large measure by the gel-forming mucin MUC2, is both barrier and sieve. Its mesh-like consistency ensnares bacteria, keeping them at least 50 μm away from the epithelial surface (Johansson & Hansson, 2016; Knoop & Newberry, 2018). Its outer mucus layer, which harbors a dense population of bacteria, shelters commensals and is continuously in the state of

shedding. And this spatial separation matters: as long as bacteria are in the outer mucus and the lumen, they tend to remain non-inflammatory (Johansson et al., 2010; Song et al., 2023) (Fig. 2).

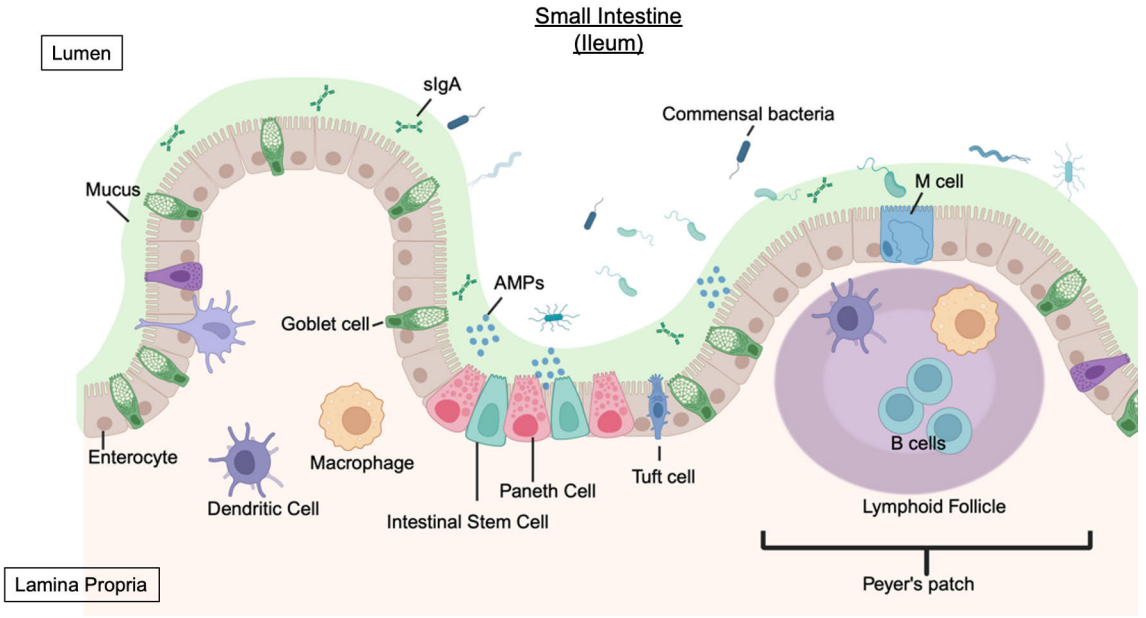
Barrier function depends on various factors: tight junctional proteins (e.g., occludin, claudins, and ZO-1) that close the junction of the epithelial cell; continuous replacement of the epithelial cells (being regulated by stem cells located in crypts) in place of the damaged ones; and normal mucus secretion and secretion of antimicrobial peptides (AMPs) (e.g., defensins, lectins and lysozyme secreted by Paneth cells in the small intestine (Clevers & Bevins, 2013)). IECs (intestinal epithelial cells) also secrete AMPs, including beta-defensins (Peterson & Artis, 2014). If these mechanisms are in working condition, the immune system is in a regulated state of ignorance or immunotolerance. But in the event of damage to the barrier of the epithelial cell, the end result is increased permeability of the intestine or "leakiness," and microbes and antigens pass across the barrier (Fig. 4). Barrier damage is typical in mucosal inflammation, it exposes immune cells located below the barrier to the microbiota, inducing immune system activation that leads to every successive disintegration of the epithelial cell lining, forming a vicious cycle (Peterson & Artis, 2014). In IBD patients, studies (Ghosh et al., 2021) described defective tight junctions and erosive lesioning in the epithelial cell lining even in ostensibly macroscopically normal tissue, implicating that dysfunction of the barrier precedes macroscopic inflammation. Pro-inflammatory cytokines like TNF-α and IFN-γ also directly decrease tight junctional proteins, increasing permeability (Kaminsky et al., 2021). TNF-α activates NF-κB–mediated upregulation of MLCK (myosin light chain kinase) and actomyosin-driven endocytic removal of occludin and claudins (Al-Sadi et al., 2016), while IFN-γ via STAT1 both represses sealing claudin transcription and induces occludin macropinocytosis (Bruewer et al., 2005; Tedelind et al., 2003), collectively compromising tight junction integrity and increasing epithelial permeability. Conversely, IL-22 (secreted by ILC3 and Th17 cells) aids in strengthening of barrier function by causing growth of the epithelial cells and goblet cells (Keir et al., 2020).

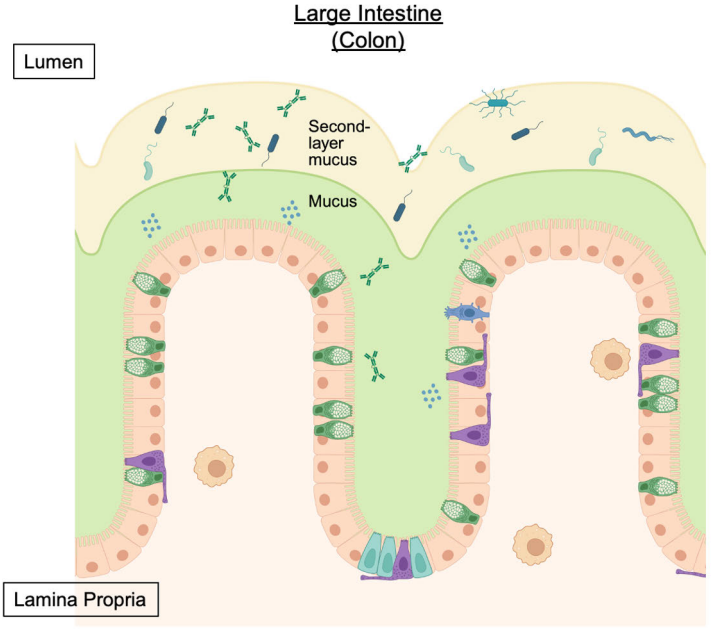
The mucus secreting goblet cells also secrete molecules like trefoil factor peptides (TFF), mucins -in particular mucin-2, Fc-binding protein (Fcgbp), and resistin-like molecules (RELM) that are involved in important intestinal defence and goblet cell response to infection (Yang & Yu, 2021). This mucus lining is especially important for protection in the colon. Johansson et al. demonstrated that mice without MUC2 (and therefore the dense inner mucus) develop a spontaneous colitis as bacteria come directly in contact with the epithelial cells and induce continuous immune activation (Arike et al., 2017; Johansson et al., 2008, 2011). Bacteria invade the crypts and induce ulcerative colitis-like inflammation in these Muc2-deficient mice without any defect in the immune system. This implies that a

defect in the mucus barrier is enough to cause colitis. Similarly, in the DSS colitis (Section 2.1.3), acute destruction of the mucus and the epithelial layer by DSS enables luminal bacteria to invade the inner mucus leading to inflammation. Hence, the barrier of the epithelium needs to be kept strong in order to prevent inflammation in the intestine.

By the point of active inflammation, the lining of the epithelial barrier is often breached, yet the body attempts to repair it. Goblet cells raise secretion of mucus in response to damage, and repair mechanisms may be initiated by epithelial cells (e.g., EGFR signaling for wound healing) (Johansson & Hansson, 2016; Yang & Yu, 2021). However, in states of chronic disease, as in IBD, inflammation itself hinders healing, leading to a cycle of damage. Medications like 5-aminosalicylic acid (5-ASA) and other biologics are reported to enhance barrier function by quelling inflammation. Other novel treatments are also in the pipeline that directly repair barrier integrity, e.g., stabilizing tight junctions or enhancing secretion of mucus (Kotla & Rochev, 2023). The importance of the epithelial barrier is also attested to by genetic studies: mutations in genes that affect lining of the epithelial integrity (e.g., in CARD15/NOD2, involving Paneth cell function and hence secretion of antimicrobial peptides (Rogler, 2007), or HNF4A, a transcription factor that regulates junctions of the lining of the epithelia (Ahn et al., 2008)) are associated with susceptibility to IBD.

In summary, the intestinal epithelial barrier is critical to intestinal homeostasis, keeping both the immune system and the microbiota in equilibrium. The gut immune system under normal conditions maintains an active state of immune tolerance through continuous low-level sampling and regulation. However, when this barrier is breached, it triggers a cascade of immune activation that can lead to disease states like IBD. Breach of mucosal barrier under susceptible conditions (risk factors mentioned in section 2.2.2) promotes mucosal inflammation, that in turn intensifies immune activation which can amplify further epithelial barrier damage. Treatment of chronic inflammation in the gut therefore often hinges on therapies that are capable of healing and maintaining the epithelial barrier to halt further rounds of damage.


Small Intestine
(Ileum)
Lumen
sIgA
Commensal bacteria
Mucus
M cell
AMPs
Goblet cell
B cells
Enterocyte
Macrophage
Dendritic Cell
Paneth Cell
Tuft cell
Intestinal Stem Cell
Lymphoid Follicle
Peyer's patch
Lamina Propria


Large Intestine
(Colon)
Lumen
Second-
layer
mucus
Mucus
Lamina Propria

◄**Figure 2. Comparative mucosal architecture of the ileum and colon.** This schematic illustrates the general organization of epithelial and immune compartments in the small (ileum) and large (colon) intestines. This figure is intended as a conceptual overview of the mucosal architecture conserved across mouse and human. **Small Intestine (Ileum):** Schematic illustrating the normal ileal epithelium and underlying lamina propria. Enterocytes form the primary absorptive surface, scattered with goblet cells that secrete mucus to cover the mucosal surface. Paneth cells at the bottom of the crypts secrete antimicrobial peptides (AMPs), while intestinal stem cells constantly replace the epithelial layer. Tuft cells are responsible for chemosensing and immunomodulation, and M cells overlie Peyer's patches to facilitate antigen sampling. Macrophages and dendritic cells reside in the lamina propria to aid in coordinating immune responses with B cells in the lymphoid follicle. Secretory IgA (sIgA) is secreted into the lumen to neutralize pathogens and maintain commensal homeostasis. **Large Intestine (Colon):** Schematic illustrating the normal colonic epithelium and its characteristic two-layer mucus barrier. Goblet cells secrete mucus that forms an inner sterile lining and an outer commensal bacteria-inhabited layer. Enterocytes absorb water and electrolytes, whereas lamina propria immune cells patrol and regulate the microbial community. Thicker mucus barrier in the colon compartmentalizes bacteria to promote a stable and protective host-microbiota relationship. Illustration created with biorender.com

2.2 Inflammatory Bowel Disease

2.2.1 Global burden and impact

Inflammatory Bowel Disease (IBD), which involves Crohn's disease (CD) and ulcerative colitis (UC), is a global health burden. In 2017, a total of 6.8 million people worldwide were living with IBD (Alatab et al., 2020). The prevalence of IBD has risen tremendously in the past decades, especially as countries become industrialized and embrace Westernized diets. Historically, the highest prevalence was found in North America and Europe, i.e., prevalence is over 0.3% of the population in these regions (Ng et al., 2017). In Europe and the United States, an estimated 1.5 million are affected, and in Europe, over 2 million, with rates reported up to 500 per 100,000 for UC and 300 per 100,000 for CD in certain Northern European countries (Ng et al., 2017). Lower incidence has, in comparison, been identified in developing world regions (e.g., as low as ~7 per 100,000 in certain regions of Africa and the Caribbean) (Alatab et al., 2020). Incidence, however, in the majority of low-incidence regions has increased over recent years. Incidence of IBD has accelerated since the 1990s, according to reporting by newly industrialized Asian nations, African countries, and those in South America (Ng et al., 2017). For instance, Taiwan and Brazil have both shown increases in rates of annual incidence of more than 10% for UC and CD in some analyses. IBD is thus becoming a disease of global rather than regional to Western populations (Kaplan & Ng, 2017). Such an epidemiologic transition would imply that lifestyle and environmental factors are primarily responsible for the development of the disease.

IBD most often develops in young adulthood and features a relapsing-remitting course with patients often residing with disease for decades. Thus, socioeconomic

costs of IBD are heavy. The continuous symptoms (abdominal pain, diarrhea, and tiredness) and need for ongoing treatment can profoundly damage quality of life and work functioning (Mehta, 2016). The majority of the patients require surgery or hospitalization throughout the disease course (Ananthakrishnan et al., 2014). IBD is also expensive in terms of healthcare costs.

The total direct and indirect cost of IBD in the United States was estimated at $14.6–$31.6 billion annually (Mehta, 2016). The cost drivers are high rates of physician visits, endoscopic procedures, expensive medications (especially biologic therapies), and surgeries for complications (Mehta, 2016). Indirect costs like work disability and absenteeism contribute to this burden. In fact, in the Global Burden of Disease study, years lived with disability (YLDs) attributable to IBD nearly doubled between 1990 and 2017, to more than 1 million YLDs worldwide (Alatab et al., 2020). With growing prevalence worldwide, IBD is a progressively demanding problem for healthcare systems and economies, and therefore the need for effective management and possibly prevention is emphasized. Worldwide, the impact of IBD is widespread and growing, and thus it is a region of high research and public health intervention priority (Alatab et al., 2020).

2.2.2 Pathophysiology and risk factors

IBD is characterized by chronic, uncontrolled inflammation of the gastrointestinal tract. Although the precise etiology remains unclear, there is a general consensus that IBD results from a dysregulated mucosal immune response to intestinal microbiota in genetically predisposed subjects, triggered by environmental stimuli (Anbazhagan et al., 2018; Friedrich et al., 2019; Neurath, 2014; Xavier & Podolsky, 2007). Basically, IBD arises as a result of an imbalance of the normal homeostatic balance between the gut immune system and the commensal flora. This immunopathogenesis includes both adaptive and innate immune components. Under normal conditions, the intestinal immune system is primed to act against pathogens while remaining tolerant to commensal bacteria. During IBD, though, the equilibrium is disrupted: harmless components of microbiota elicit a disproportionate immune reaction that leads to tissue damage as well as long-term inflammation (Abraham & Cho, 2009). More details on the flow of events, mechanisms of immune activation and immune components involved are detailed in section 2.2.2.3. Studies in patients also support this model, e.g., gut microbes are believed to induce the inflammation, as indicated by the finding of dysbiosis (microbial population changes) in IBD (Caruso et al., 2020). Patients with Crohn's ileitis have reduced gut bacteria diversity, loss of commensal *Firmicutes* and elevated levels of pathogenic bacteria like adherent-invasive *Escherichia coli* (Darfeuille-Michaud et al., 2004; Frank et al., 2007). This dysbiosis is thought to both induce and sustain the inflammatory state.

2.2.2.1 Genetic Factors

Genome-wide association studies have implicated over 240 risk loci for IBD, the majority of which are shared between CD and UC (Huang et al., 2017; Mirkov et al., 2017; Peters et al., 2017). These genes highlight key pathways in IBD pathogenesis, including innate immune sensing (e.g., NOD2), adaptive immunity (e.g., IL23R), epithelial barrier function, autophagy, and cytokine signaling. NOD2 gene is an excellent example: NOD2 variants were among the first genetic risk factors identified for Crohn's disease (Hugot et al., 2001). NOD2 encodes for an intracellular pattern recognition receptor that allows intestinal cells to detect bacterial peptidoglycan. Certain mutations of NOD2 associated with Crohn's disease interfere with this sensing and have been found to be associated with reduced secretion of antimicrobial defensins by Paneth cells in the ileum (Wehkamp et al., 2005). This defect would allow bacteria easier entry and cause inflammation (Caruso et al., 2020; Noor et al., 2020). The other risk genes (e.g., ATG16L1 and IRGM) suggest defective autophagy and bacterial clearance in Crohn's (Massey et al., 2008; Mehto et al., 2019; Salem et al., 2015), and then some risk genes in ulcerative colitis point to barrier function and cytokine pathways (e.g., ECM1, IL-10) (Jostins et al., 2012). While the genetic input is clear, there is no causative gene, rather, each confers moderate risk, and environmental stimuli are required for disease initiation.

2.2.2.2 Role of Environmental Triggers

Environmental factors play a role in the risk and course of inflammatory bowel disease (IBD), and among the most well-established is cigarette smoking. Smoking has a striking dichotomy between ulcerative colitis (UC) and Crohn's disease (CD): in CD, smoking roughly doubles disease risk and is associated with poorer outcomes, e.g., higher frequency of fistulas, higher likelihood of surgery, and higher rates of post-surgery recurrence, while in UC, smoking appears to be protective, with current smokers having roughly half the risk of non-smokers (Kobayashi et al., 2020). Furthermore, many UC patients develop the disease shortly after having given up smoking; indeed, cessation leads to a sustained increase in UC risk for up to 10 years, possibly due to differential effects of nicotine or other tobacco constituents on immune responses and epithelial function in these diseases (Bernstein et al., 2016). Other environmental factors such as geographical, social, and dietary factors aside from smoking are also relevant. A "Western" diet that is high in animal protein, saturated fat, and sugar and low in fiber has been linked with higher IBD risk, whereas diets rich in fruit, vegetables, and n-3 polyunsaturated fatty acids are protective (Ananthakrishnan, 2015; Lewis & Abreu, 2017). For instance, a prospective study found that a high dietary intake of long-chain n-3 PUFAs is associated with approximately a 28% reduced risk of UC, while high dietary intake of trans-fats

appears to increase it. Further, dietary fiber may be beneficial for the gut by energizing beneficial microbiota and producing anti-inflammatory metabolites; fiber ligands can activate the aryl hydrocarbon receptor (AhR) on intestinal immune cells, thereby enhancing mucosal defense (Ananthakrishnan, 2015). Other risk modulators include appendectomy (decreasing UC risk especially if performed in childhood but not in CD (Andersson et al., 2001; Gardenbroek et al., 2012; Kaplan et al., 2008)), psychosocial stress (Bernstein, 2016), vitamin D deficiency (Ananthakrishnan AN et al., 2012 & 2013), and exposure to drugs such as NSAIDs or antibiotics. Antibiotic exposure, particularly during early life, has been associated with increased IBD risk. Antibiotics can alter the gut microbiota composition, reducing microbial diversity and immune-microbial crosstalk. Repeated or broad-spectrum antibiotic exposure has been demonstrated by numerous studies to increase the incidence of both UC and CD (Shaw et al., 2010; Ungaro et al., 2014). This disruption can predispose the mucosa to abnormal immune activation. This pattern of multifactorial influence highlights the fact that, although CD and UC share an extensive array of genetic and immunological features, certain environmental interactions can steer the courses of the diseases onto divergent clinical trajectories.

2.2.2.3 Pathological Features and Immune System Activation

Ulcerative colitis and Crohn's disease represent two ends of the IBD spectrum with some overlapping and many distinct pathological features (Fig. 3).

Ulcerative colitis is an inflammatory disease localized to the colon (large intestine). It involves the rectum and extends proximally in a continuous manner to affect a variable length of colon (Fig. 3). Inflammation in UC is limited to the mucosa and the uppermost part of the submucosa of the bowel wall. This leads to the formation of superficial ulcers of the colonic mucosa and the development of pseudopolyps (enclaves of regenerating mucosa between patches of ulceration). Because damage does not typically extend all the way through the bowel wall, complications like fistulas or perforation are uncommon with UC. Bloody diarrhea, urgency, and crampy abdominal pain occur in most patients with ulcerative colitis. Microscopically, UC is defined by crypt architectural disruption, neutrophil crypt abscesses (in colonic glands), and goblet cell depletion (which reflects impaired mucin production).

By contrast, Crohn's disease can affect any part of the gastrointestinal tract from mouth to anus, though in most cases terminal ileum and colon are affected (Fig. 3). Crohn's disease is typically characterized by non-contiguous inflammation, with discoid areas of abnormal mucosa (so-called "skip lesions") separating areas of relatively normal bowel. One of the features of Crohn's is that it is transmural - the inflammation extends the entire thickness of the intestine wall. Consequently, Crohn's disease can cause extensive ulcerations, fissures, and fistula tracts that go to

the skin or adjacent organs. Stricture due to fibrosis of the bowel wall is another common complication of chronic Crohn's. Microscopically, Crohn's disease is defined by the presence of granulomas (aggregates of epithelioid macrophages) in the bowel wall, a sign of a chronic cell-mediated immunity.

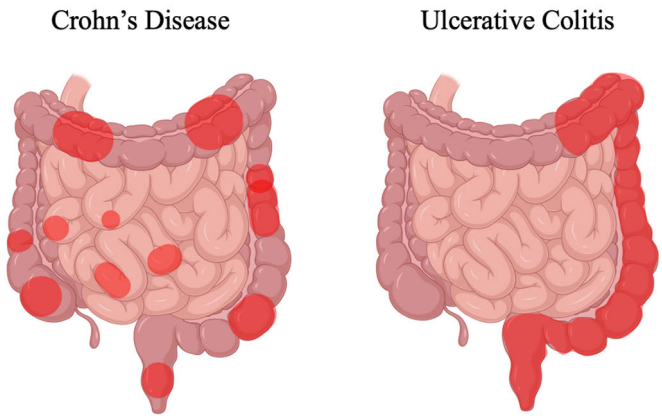


Figure 3. Inflammation locations in Crohn's disease and Ulcerative colitis. Crohn's Disease with skip lesions in small intestine and large intestine (left). Ulcerative Colitis with continuous inflammation in the colon. Illustration created with biorender.com

Ulcerative colitis (UC) and Crohn's disease (CD) are thought to arise from an inappropriate immune response to intestinal microbiota in genetically susceptible hosts, as mentioned previously. A breakdown of the mucosal barrier (epithelial tight junction defects, mucus layer alterations) allows luminal antigens to invade the lamina propria, triggering innate immune activation (Fig. 4). Antigen-presenting cells such as dendritic cells and macrophages first respond to microbial components using their pattern recognition receptors (e.g., NOD2, TLRs), thereby releasing cytokines like interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), and IL-23 (Lee et al., 2018). Then, this innate cytokine environment drives the differentiation of the naïve $CD4^+$ T cells into pro-inflammatory effector subsets. In Crohn's disease, dendritic cell production of IL-12 and IL-18 skews T cells toward a T helper type 1 (Th1) phenotype, yielding high levels of interferon-γ (IFN-γ) and activation of macrophages; concurrently, IL-23 promotes expansion of Th17 cells that secrete IL-17 and IL-22 (Fig. 4). On the other hand, Ulcerative colitis, is less Th1-dominant and instead tilts to a Th2-like immune profile: mucosal innate cells (including dendritic and innate lymphoid cells) produce IL-33, which upregulates IL-5 and IL-13 as part of an atypical Th2 response in the colon (Torres et al., 2017; Ungaro et al., 2017). Another T cell subset, Th9, has been implicated in UC-specific pathology, as mentioned earlier; IL-9–producing Th9 cells are enriched in UC and drive inflammation by impairing the

epithelial barrier and wound healing. Indeed, Gerlach et al. (2014) showed that mice lacking the Th9 regulator PU.1 or treated with anti-IL-9 are protected from colitis, highlighting IL-9's pathogenic role in UC (Gerlach et al., 2014). In both forms of IBD, a failure of regulatory mechanisms (e.g., regulatory T cells producing IL-10, or tolerogenic cytokines like transforming growth factor-β) permits unrestrained inflammation. Notably, deficient IL-10 signaling precipitates severe, early-onset IBD in humans, and lamina propria TGF-β1 levels are paradoxically elevated in UC but reduced in CD (Ihara et al., 2017), suggesting an imbalance in counter-regulatory versus fibrogenic responses. These events create a self-developing inflammatory cascade that leads to chronic tissue injury and ulceration. The intestinal mucosa ultimately becomes infiltrated with neutrophils, macrophages, and T cells, perpetuating cycles of damage (e.g., crypt abscesses in UC and transmural granulomas in CD) and disrupting normal gut function.

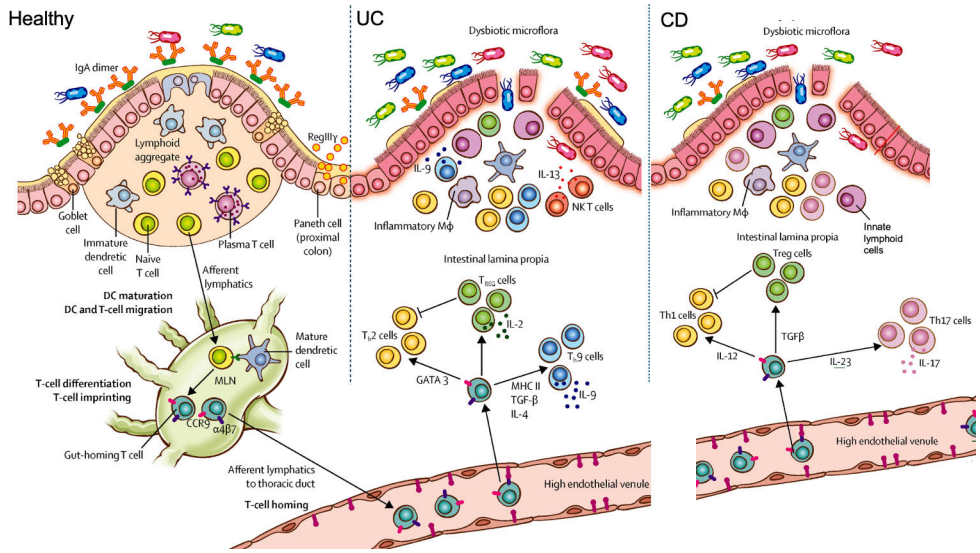


Figure 4. Schematic drawing of normal colon (left), ulcerative colitis (UC; center), and Crohn's disease (CD; right). In healthy states, an intact mucus layer, homeostatic gut microbiota, and secretory IgA prevent excess microbial contact, while dendritic cells (DCs) and other lamina propria (LP) antigen-presenting cells (APCs) promote immune tolerance by regulating T cell differentiation in mesenteric lymph nodes. In UC, epithelial barrier dysfunction and dysbiotic microbiota facilitate trans-epithelial passage of antigens to induce Th2/Th9-dependent inflammation; hallmark cytokines (e.g., IL-5, IL-13, IL-9) activate innate cells (neutrophils, innate lymphoid cells, NKT cells) and cause mucosal damage and ulcerations. In CD, dysbiosis and innate defect in pattern recognition (by NOD2, IL-23R) impose Th1/Th17-cell differentiation (IFN-γ, IL-17, IL-22) causing granulomas, transmural inflammation, and fibrotic complications. Figure is modified from Ungaro et al., 2017 and Torres et al., 2017, licensed for reuse under Copyright Clearance Center's RightsLink® service.

Although there are differing immune profiles, UC and CD show some overlapping inflammatory pathways alongside their key distinctions. Crohn's disease is classically characterized by a Th1/Th17-driven response (Fig. 4): affected tissues contain abundant IFN-γ and IL-17, and the combination of Th1 and Th17 cytokines (e.g., IFN-γ, IL-17, IL-22, TNF-α) develops granulomatous and also causes transmural inflammation. Consistent with this, pathogenic innate lymphoid cells of the ILC1 and ILC3 subsets (which produce IFN-γ and IL-17/IL-22, respectively) are also found to be enriched in Crohn's lesions, mirroring the Th1/Th17 polarization of adaptive cells. Genetic mutations, as stated earlier, increase Th1/Th17 axis in CD, and risk mutant forms of genes such as NOD2 (pattern recognition) and IL23R increase cellular reactivity to various microbial stimuli (Neurath, 2014). Ulcerative colitis, in contrast, is marked by Th2/Th9-polarized immune response localized to colonic mucosae (Fig. 4). Th2 cytokines, IL-13 (usually derived from mucosal NK-T cells) and IL-5 in UC and participate in epithelial damage and goblet cell loss, while IL-9 derived from Th9 further handicaps barrier functions (Gerlach et al., 2014). UC lesions typically lack the granulomas of Crohn's and instead show superficial ulceration with prominent neutrophil infiltration; accordingly, neutrophil-mobilizing chemokines like IL-8 are markedly upregulated in active UC. Common ground exists in the inflammatory circuitry of UC and CD. Both diseases exhibit elevated levels of TNF-α, a central pro-inflammatory mediator that drives recruitment of immune cells and amplifies cytokine cascades and both show increased IL-23/Th17 activity in inflamed tissues. These shared pathways explain why broad-spectrum biologics and small molecules can treat both conditions. For example, anti-TNF therapies induce remission in a significant fraction of UC and CD patients, and blocking the IL-12/IL-23 p40 subunit with ustekinumab has proven effective in Crohn's (Sandborn et al., 2012, 2018) and recently in UC, reflecting the pathogenic role of the IL-23–Th17 axis in both. In a similar manner, integrin antagonists that prevent lymphocyte gut homing (e.g., vedolizumab targeting α4β7 integrin) benefit both UC and CD by reducing immune cell infiltration (Feagan et al., 2013; Sandborn et al., 2013). However, divergent therapeutic outcomes highlight differences in cytokine dependencies, between UC and CD: for instance, IL-17A neutralization failed to help Crohn's disease patients and even exacerbated disease in clinical trials, consistent with findings that IL-17 contributes to mucosal barrier defense (Hueber et al., 2012). Conversely, blockade of IL-13 (a pivotal Th2 cytokine in UC) has been explored as a UC-specific strategy in an effort to reduce epithelial injury and colonic inflammation (Hoving, 2018). In summary, UC and CD represent two ends of an immunologic spectrum in inflammatory bowel disease, one biased toward a Th2/IL-9–mediated mucosal inflammatory process, the other toward a Th1/Th17-driven transmural inflammation, yet they overlap in fundamental mechanisms of chronic intestinal inflammation.

2.2.3 DSS-induced colitis mouse model of IBD

Mouse models are crucial for probing the IBD pathogenesis and for the development of therapeutics. Among the most frequently used is the dextran sulfate sodium (DSS)–induced colitis model, which mimics mainly ulcerative colitis (Fig. 5) (first published mouse model of DSS-induced colitis, Okayasu et al., 1990). This section discusses the acute DSS-induced colitis model, in which inflammation is rapidly induced by short-term exposure to DSS. Chronic DSS colitis models, involving cyclic or prolonged DSS administration, are also used to mimic relapsing-remitting IBD phenotypes. DSS is a sulfated polysaccharide that, when administered in drinking water to rodents, causes acute colonic inflammation. This model is popular because of its reproducibility and simplicity – a short treatment of DSS causes lesions to the mouse distal colon mucosa (Laroui et al., 2012; Mizoguchi et al., 2013). Mechanistically, DSS first disrupts intestinal epithelial barrier integrity, and then causes local innate inflammation (Laroui et al., 2012). The large negatively charged, water-soluble polysaccharide, DSS, have been found to cause direct injury to the epithelial monolayer, potentially by forming nanometer-size complexes with lipid membranes (Laroui et al., 2012), leading to increased intestinal permeability. This leads to movement of gut flora and their derivatives across into the mucosa, initiating an immune response dominated by innate cells. A typical feature of DSS colitis is damage to colonic crypts with loss of goblet cells, as well as infiltration of neutrophils and monocytes into lamina propria and submucosa. Clinical manifestations (e.g., weight loss, diarrhea, rectal bleeding) and histologic features of colitis become evident on day 3-5 of DSS treatment and peak at one week (Laroui et al., 2012). Intensity of DSS colitis could also be protocol- and strain-dependent in mice.

Genetic susceptibility to DSS damage varies considerably between inbred strains (Mähler et al., 1998). For example, C3H mice are highly susceptible to develop deep ulcers and inflammation, while C57BL/6 or Balb/c strains show middle-grade inflammation which spontaneously recovers when DSS is withdrawn (Mähler et al., 1998). Such variation would imply that factors (aside from the adaptive immune system) influence epithelial regeneration and innate immune responsiveness. Accordingly, DSS concentration and duration of exposure per strain must be optimized (usually 2-5% DSS in drinking water for 5-7 days, and then plain water only). Furthermore, studies show that DSS molecular weight and origin could impact results, and proper preparation is required to avoid batch-to-batch variation (Perše & Cerar, 2012). Such precautions aside, the DSS model remains a robust method for IBD research due to its faithful mimicry of ulcerative colitis-like pathology. It induces superficial colonic ulceration, crypt abscesses, and bleeding, all of which are comparable to human ulcerative colitis (Johansson et al., 2010; Laroui et al., 2012). In particular, research using DSS has shed light on the fundamental role of the

epithelial barrier in colitis. Johansson et al., 2010, have demonstrated that feeding DSS rapidly alters the colonic mucus layer: within minutes otherwise impermeable inner mucus is permeable, and in a few hours luminal bacteria move to the epithelial surface. This bacterial invasion is preceded by inflammatory cell infiltration, implying that disruption of the barrier is the starting point in DSS colitis.

The DSS model, represents a two-hit process: chemical insult to epithelium followed by microbial-stimulated inflammation. It is therefore a useful model to investigate innate immunity, epithelial function, and possibly therapeutic agents (e.g., agents promoting mucosal healing or cytokine modulation). But extrapolating too directly to human IBD needs to be done with caution, since DSS colitis is an acute chemical-induced injury (with features of innate immunity), and it lacks the adaptive immune component and transmural granulomas of Crohn's disease.

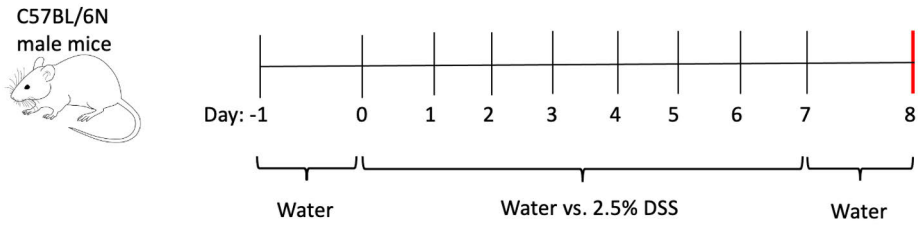


Figure 5. Schematic representation of oral dextran sulfate sodium (DSS)-induced acute colitis mouse model. 2.5% DSS in water is given daily for 7 days and then one day of plain water. On 8th day, the mice are sacrificed, and samples are collected. Mice are monitored every day from a day before the DSS-treatment is started. This includes measuring weight, and quantifying stool consistency (diarrhea) and rectal bleeding. Illustration created with Microsoft PowerPoint.

2.3 Salmonellosis

Salmonellosis is an infection caused by *Salmonella enterica*, a Gram-negative rod-shaped bacterium, in humans, which usually leads to acute gastroenteritis (inflammation of the intestine characterized by diarrhea, fever, and abdominal pain). Most of the infections are communicated by nontyphoidal *Salmonella* serovars (e.g., *S. enterica* serovar Typhimurium) responsible for causing local self-limiting infection, while typhoidal serovars like *S. enterica* serovar Typhi induce systemic typhoid fever. In the lab, an *S. enterica* serovar Typhimurium infection in mice is used widely as an animal infection model for understanding *Salmonella*-mediated gut inflammation (Hapfelmeier & Hardt, 2005; Herzog et al., 2023).

2.3.1 Incidence and Prevalence

It was estimated in 2006 that non-typhoidal *Salmonella* causes around 93.8 million cases of gastroenteritis globally each year, resulting in approximately 155,000 deaths annually (Besser, 2018; Majowicz et al., 2010). According to the Global Burden of Disease 2019 estimates, invasive non-typhoidal *Salmonella* (iNTS) disease affected approximately 594,000 people worldwide (with a range of 486,000 to 718,000 cases), leading to around 79,000 deaths (ranging from 43,000 to 124,000) and contributing roughly 6.11 million disability-adjusted life years (DALYs) globally (with an uncertainty interval of 3.32 to 9.71 million) (Stanaway et al., 2019). This ranks *Salmonella* as one of the top diarrheal causes in the world. The World Health Organization (WHO) ranks *Salmonella* among the four most important diarrheal pathogens (after rotavirus, *Shigella*, and pathogenic *E. coli*) (Lamichhane et al., 2024). Regionally, salmonellosis affects both the industrialized and developing worlds but in different contexts. In developed countries, *Salmonella* is a major driver of foodborne outbreaks and sporadic illness linked with contaminated food sources. For example, in the U.S., the Centre for Disease Control and Prevention (CDC) approximates that non-typhoidal *Salmonella* infects about 1.35 million, hospitalizes 26,500, and kills 420 each year (cdc.gov). This makes it the second most frequent bacterial cause of foodborne disease in the U.S. (after *Campylobacter*). The financial cost in the United States alone is significant: *Salmonella* infections amount to a total of around $3.3 billion annually in direct and indirect costs (Hoffmann et al., 2012). In Europe, surveillance of outbreaks shows thousands of laboratory-confirmed cases every year, with *Salmonella* consistently being identified in eggs, poultry, and fruit. Salmonellosis is the second most commonly notified gastrointestinal disease in the EU/EEA, and one of the major causes of food-borne outbreaks. In 2022, 65,967 laboratory-confirmed cases of salmonellosis were reported in the EU/EEA, out of which 81 were fatal, a rate of 15.5 cases per 100 000 population (Annual Epidemiological Report 2022, ECDC, https://www.ecdc.europa.eu/sites/default/files/documents/SALM_AER_2022_Report.pdf). In low- and middle-income countries, *Salmonella* presents a dual challenge: nontyphoidal strains cause diarrheal disease, particularly in young children, and typhoidal strains (such as *Salmonella* Typhi) cause systemic typhoid fever in areas with inadequate clean water and sanitation. In sub-Saharan Africa, iNTS infections (which can cause bacteraemia in immunocompromised individuals, including HIV-infected or severely malnourished individuals) are a leading cause of mortality (Reddy et al., 2010).

One of the main reasons for the common prevalence of *Salmonella* is that it takes place along the entire "farm-to-fork" continuum of food production (Lamichhane et al., 2024). *Salmonella* naturally resides the intestines of most wild and domestic animals, and food products can be contaminated at some point or other. Transmission

to humans typically occurs through the consumption of contaminated food and/or water. Poultry and poultry products (meat chicken, eggs) are the most common sources of *Salmonella*, since the bacteria can colonize on the ovaries of the hens (internal egg contamination) or on the surface of meat during processing (Kimminau et al., 2021, 2022). Beef, pork, dairy products, and even fruits and vegetables (often via manure-contaminated irrigation water) are other vehicles (Ehuwa et al., 2021). Poor kitchen hygiene and cross-contamination can spread *Salmonella* from raw foods to other items. Given this ubiquity, sporadic cases and outbreaks of salmonellosis occur worldwide. More than 2,600 *Salmonella* serovars have been described (Issenhuth-Jeanjean et al., 2014), but few (e.g., *S. enterica* serovar Typhimurium and serovar Enteritidis) cause most cases of human gastroenteritis.

A new challenge in salmonellosis epidemiology is the rise of antimicrobial resistant *Salmonella*. Multi-antibiotic-resistant isolates (e.g., *S.* Typhimurium DT104 clone) have led to outbreaks, since late 90s, and make therapy difficult, especially for invasive infections. This has led many countries to monitor *Salmonella* in food animals and limit unnecessary antibiotic use. Despite enhanced food safety through modernization, *Salmonella* remains a virulent pathogen due to its persistence (H.-M. Chen et al., 2013; Helms et al., 2005; Mølbak et al., 1999; Novoa Rama et al., 2022).

2.3.2 Mechanism of Infection and Gut Immune Responses

Salmonella are facultative intracellular pathogens that have evolved sophisticated mechanisms to invade the host and thrive within the gut environment. *Salmonella enterica* serovar Typhimurium (hereafter referred as STm) is a well-established model for studying gut infections. The disease process typically begins with the ingestion of contaminated food or water. The bacteria then pass through the acidic stomach and reach the intestine, where they initiate infection in the distal ileum and colon. A critical early step is adherence and invasion of the intestinal epithelium. STm can adhere to the mucus and epithelial surface and preferentially invade specialized epithelial cells called M cells (microfold cells) in the Peyer's patches of the small intestine. These M cells are designed to sample antigens and deliver them to underlying immune cells, which STm utilizes as a portal to the gut mucosa. STm also invades absorptive enterocytes by inducing its own uptake. To achieve this, STm releases a dedicated set of virulence factors encoded on Salmonella Pathogenicity Island 1 (SPI-1). The same SPI-1 also encodes a Type III Secretion System (T3SS-1), which is essentially a molecular needle that injects bacterial effector proteins into host cells (Fattinger et al., 2021; Galán, 2021). These effector proteins then trigger cytoskeletal rearrangements in the host cell, causing membrane ruffling and the eventual engulfment of the bacteria (a process of forced

phagocytosis with a non-phagocytic cell). This active invasion mechanism allows STm to translocate across the epithelium. Once across, STm is phagocytosed by resident macrophages and dendritic cells in the lamina propria. Inside phagocytes, STm expresses another suite of effectors via a second Type III Secretion System (T3SS-2) encoded on SPI-2. T3SS-2 effectors remodel the phagosome, creating a specialized Salmonella-containing vacuole (SCV) in which the bacteria can replicate. These effectors help STm survive the hostile intracellular environment by interfering with vesicle trafficking, inhibiting oxidative burst, and modulating cell signaling (Galán, 2021; Srikanth et al., 2011; Waterman & Holden, 2003). The net effect is that STm can persist inside macrophages, which can carry the bacteria through the lymphatics to systemic sites (in typhoidal serovars) or remain within gut-associated phagocytes (in gastroenteritis).

The host's immune system detects STm invasion quickly, especially in the case of non-typhoidal strains. STm possesses molecules recognized as pathogen-associated molecular patterns (PAMPs), for example, lipopolysaccharide (LPS) in its outer membrane and flagellin in its flagella. Intestinal epithelial cells and immune cells (macrophages, dendritic cells) express pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) that bind these PAMPs (Creagh & O'Neill, 2006; Jessen et al., 2014). TLR4 on the cell surface and within endosomes recognizes STm LPS (Chow et al., 1999; Medzhitov et al., 1997) and TLR5 while TLR5 on the epithelial cell surface recognizes flagellin (Hayashi et al., 2001). Activation of TLRs accelerates the acidification process within harboring phagosomes, and blocking this acidification stops SPI 2 from being triggered (Arpaia et al., 2011). Arpaia N. et al. (2011) also demonstrated that STm depends on signals from the innate immune system to fine tune the expression of virulence genes critical for surviving inside cells, replicating, and spreading systemically. Additionally, intracellular sensors like NLRC4 (an NLR) can detect flagellin or components of the T3SS inside the host cell cytosol (Naseer et al., 2022). Engagement of these PRRs triggers multiple signaling cascades like those of NF-κB and MAPK, resulting in the synthesis of pro-inflammatory cytokines and chemokines (Patel & McCormick, 2014). For instance, TLR4 detection of STm LPS or NOD1/2 detection of peptidoglycan in the epithelial layer produces IL-8, a chemokine that strongly attracts neutrophils to the site of infection. Neutrophil recruitment is emphasized in STm colitis: the infected intestinal mucosa experiences acute inflammation with massive polymorphonuclear leukocyte infiltration. These neutrophils, along with macrophages, attempt to contain the pathogens and clear them. They phagocytose the bacteria and secrete reactive oxygen species, antimicrobial peptides, and enzymes. This response is critical in controlling the infection; however, excessive neutrophil activity causes concomitant destruction of intestinal tissues leading to

epithelial breakdown and excessive secretion of fluids that give rise to diarrhea (Kurtz et al., 2017; Loetscher et al., 2012).

Salmonella-induced gut immune response is a complex orchestration between both innate and adaptive immunity, and needs a harmonised synchrony during the acute phase. Innate cells are of course centre stage: neutrophils, macrophages, dendritic cells, and innate lymphoid cells all get their turn in containing the infection, as mentioned above. Macrophages release inflammatory cytokines like TNF-α, IL-6, and IL-12 that help in the activation of other immune cells. Dendritic cells that have engulfed *Salmonella* or antigens of *Salmonella* will migrate into the mesenteric lymph nodes and stimulate T cells. An adaptive immune response is induced in days (Cummings et al., 2009). IL-12 and IL-18 activation induce Th1-type CD4 T cells, which differentiate and produce IFN-γ. IFN-γ activates macrophages to enhance their microbicidal functions (e.g., inducing nitric oxide synthase for nitric oxide production). This Th1 response is crucial in eliminating intracellular *Salmonella* that persist inside macrophages. CD8 T cells also contribute by eliminating infected cells. Meanwhile, B cells produce antibodies (specifically IgA in the intestinal lumen and IgG systemically) against antigens of *Salmonella*, which may be utilized to neutralize or to opsonize the bacteria (Cummings et al., 2009). In most cases of non-typhoidal *Salmonella* infection, this dual innate and adaptive response will resolve the infection within a week or two, and the patient recovers. In severe infections or in immunocompromised individuals, medical treatment with antibiotics may be necessary to assist bacterial clearance. What's fascinating is that the very same symptoms *Salmonella* causes, vomiting and diarrhea, which also serve as a method of transmission, making it easier to spread the organism to new hosts. Therefore, from the pathogen's perspective, inducing a self-limiting yet highly inflammatory infection is a viable method of propagation.

2.3.3 Streptomycin pre-treatment mouse model of infection

Studying *Salmonella* pathogenesis *in vivo* has been greatly advanced by the development of the streptomycin pre-treated mouse model of *Salmonella enterica* serovar Tyhphimurium colitis, as first described by Barthel et al. in 2003 (Barthel et al., 2003) (Fig. 6). In natural conditions, adult mice infected orally with STm do not exhibit the intestinal inflammation seen in humans; instead, the bacteria tend to colonize Peyer's patches and then cause a systemic, typhoid-like illness in mice (with colon inflammation being mild or absent). This discrepancy is partly because the native mouse gut microbiota prevents STm from efficiently colonizing the gut lumen and invading the mucosa (Clark et al., 1994; Jones et al., 1994; Penheiter et al., 1997; Rescigno et al., 2001; Vazquez-Torres et al., 1999). Barthel and colleagues in 2003 found that by giving mice a dose of streptomycin (an antibiotic) about 24 hours prior

to infection, they could disrupt the normal microbiota enough to allow STm SL1344 to vigorously infect the cecum and colon. The streptomycin pre-treatment model produces an acute colitis in mice that closely resembles human *Salmonella* gastroenteritis in terms of pathology and immune response. In streptomycin-treated mice, oral challenge with STm SL1344 leads to rapid colonization of the cecum and colon (as the competing commensal bacteria have been knocked down). Within a day, the mice develop notable intestinal inflammation: the cecal and colonic tissue show edema, submucosal swelling, epithelial ulceration, and massive neutrophil influx. Inflammatory marker intercellular adhesion molecule-1 (ICAM-1) was found strongly induced in the colonic mucosa, reflecting activation of the innate immune response.

It is important to note that Barthel et al. showed that this colitis is dependent on STm's virulence factors, validating that the model truly engages the pathogenic mechanisms of the bacterium. They showed that mice infected with a STm SL1344 mutant (SB161) lacking a functional SPI-1 T3SS (the invasion apparatus) failed to develop a strong colitis, thereby indicating that active invasion of the epithelium via T3SS-1 is required to trigger the intestinal inflammation. Additionally, by using genetically modified mice, the model has shown that organized gut-associated lymphoid tissues (such as Peyer's patches and mesenteric lymph nodes) are not necessary for acute *Salmonella* colitis. Even mice lacking all lymph nodes (LTβR-knockout mice) still developed colitis after streptomycin and *Salmonella* infection. This underscores that the acute inflammatory response is primarily driven locally by innate immunity rather than requiring primed adaptive responses in lymphoid structures (at least in the early stage). The streptomycin pre-treatment model has since become a standard tool to study *Salmonella*-host interactions, allowing for the probe of both pathogen and host factors in a controlled setting. For example, it enables testing of *Salmonella* mutants (to see which genes are needed for causing colitis) and testing of host gene knockouts (to see which immune pathways are critical for defense or pathology).

The model holds high relevance to human disease as it duplicates several significant aspects of *Salmonella* colitis, including neutrophil-dominant inflammation, cytokine production, tissue pathology, and bacterial growth kinetics similar to those seen in human intestinal biopsies in Salmonellosis. The model reproduces the importance of microbiota for infection resistance. Gut microbiota in healthy mice induces "colonization resistance" against infection within the gut that is disrupted by antibiotic perturbation. This has especially important clinical relevance, as individuals who recently were on antibiotics would be more vulnerable to infections such as *C. difficile*, due to antibiotic-induced microbiota disruption. Although susceptibility to pathogens like *Salmonella* may also be enhanced, the mechanisms are pathogen-specific and involve distinct host–microbiome interactions.

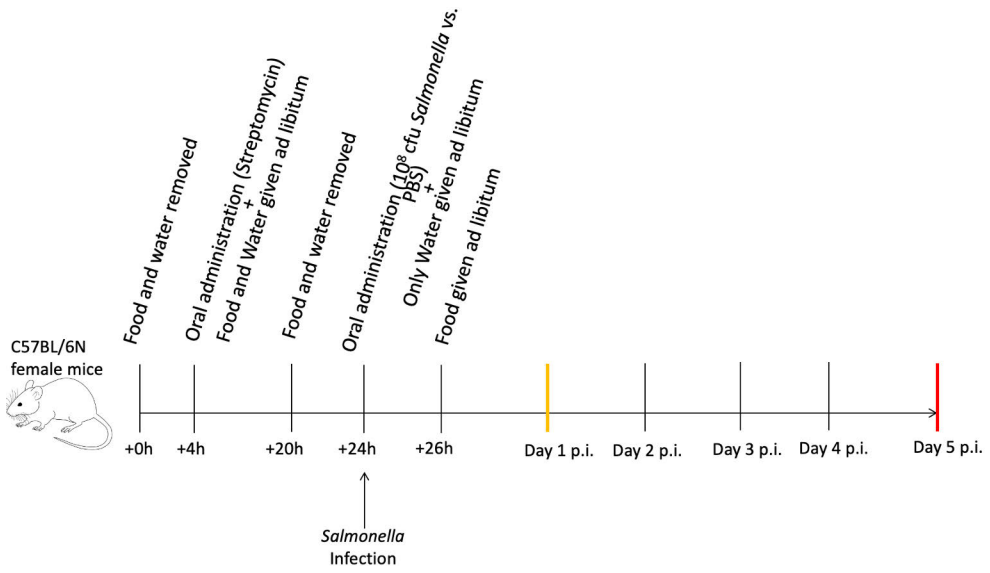


Figure 6. Schematic representation of the streptomycin-pretreatment *Salmonella* Typhimurium infection model in C57BL/6N female mice, adapted from Barthel et al. 2003. Briefly, food and water are removed for a defined period, followed by oral administration of streptomycin. After an additional period of withholding food and water, mice receive an oral gavage of *Salmonella* Typhimurium. Mice are then provided food and water ad libitum. In the present study, mice were sacrificed for sample collection on Day 1 post-infection (p.i.; indicated by the yellow bar) and Day 5 p.i. (red bar) to assess early and later stages of infection. Illustration created with Microsoft PowerPoint.

2.4 ADP-ribosylation and PARP14

2.4.1 ADP-ribosylation and Catalyzing Proteins

ADP-ribosylation is a reversible post-translational modification (PTM) with ubiquitous impact on cellular function. It was first reported in the 1960s on nuclear proteins, and years of research have revealed that it is indeed a conserved regulatory mechanism that is observed in all kingdoms of life (from bacteria to humans) (Aravind et al., 2014; Chambon et al., 1963; Kraus, 2015; Pazzaglia & Pioli, 2019). In an ADP-ribosylation reaction, the enzyme (an ADP-ribosyltransferase, or ART) transfers an ADP-ribose unit from NAD^+ to single acceptor residues (e.g., glutamate, aspartate, arginine, cysteine, or serine) on a target protein (or other substrate) (Fig. 7A). This can occur once (mono-ADP-ribosylation, MARylation) or iteratively to form branched or linear chains of ADP-ribose (poly-ADP-ribosylation, PARylation) on the target (Fig. 7B). These large ADP-ribose additions alter the properties and interactions of the target molecule, thereby controlling its function. In fact, ADP-ribosylation has been implicated in fundamental processes like DNA damage repair,

transcription and chromatin control, RNA processing, cell cycle control, stress granule formation, immune response, and cell death programs (Bai, 2015; Gupte et al., 2017). For example, poly(ADP-ribose) signaling by nuclear PARP enzymes is a well-known mechanism to recruit DNA repair factors to sites of strand breaks, linking ADP-ribosylation to genome stability and cell survival. Likewise, recent advances have connected ADP-ribosylation to pathways such as NF-κB, JAK/STAT, Wnt/β-catenin, MAPK, and PI3K-AKT signaling, underscoring that this modification can influence many intracellular signaling cascades (Boehi et al., 2021). ADP-ribosylation is thus recognized as an important regulatory strategy that allows cells to rapidly respond to stress signals, damage, or pathogen attack by modifying key proteins in a transient and reversible manner (Gupte et al., 2017).

2.4.1.1 Writers, Erasers and Readers

Enzymes (“writers”) of ADP-ribosylation, i.e., two main families of enzymes catalyze ADP-ribosylation in mammalian systems: the cholera toxin-like extracellular ARTs and the diphtheria toxin-like ARTs. The ecto-ARTs (cholera toxin-like ARTs or ARTCs) are usually membrane-associated or secreted enzymes that act outside or on the cell surface; they use extracellular NAD^+ (often released during cell damage or stress) to mono-ADP-ribosylate target proteins on the cell membrane or in the extracellular space. In contrast, the intracellular PARP family (also known as diphtheria toxin-like ARTs, or ARTDs) operate within the nucleus and cytoplasm (Hottiger et al., 2010). The PARP (Poly-ADP-ribose polymerase) family in humans consists of 17 members (PARP1–PARP16, plus the tankyrases PARP5a/5b) that share a homologous catalytic domain. Notwithstanding the historical designation "poly-ADP-ribose polymerases," it is currently appreciated that just a few of the enzymes (in particular, PARP1, PARP2, and tankyrases PARP5a/5b) are proficient in the synthesis of poly(ADP-ribose) polymers, while most of the PARPs catalyze mono-ADP-ribosylation or possess very poor polymerase activity (Morone & Grimaldi, 2024). PARP1 and PARP2 are self-PARylating DNA damage sensors activating DNA repair, while many others (e.g., PARP7, PARP10, PARP12–16) are mono-ARTs that transfer a single ADP-ribose unit onto targets (Dhoonmoon & Nicolae, 2023; Di Paola et al., 2022; Grimaldi et al., 2022; Mehrotra et al., 2011; Nicolae et al., 2015; Palavalli Parsons et al., 2021; Rodriguez et al., 2021; Yu et al., 2005). Some PARPs (e.g., PARP13) are not active as catalysts but may still serve as "readers" or scaffold proteins in ADP-ribose signal transduction pathways (Teloni & Altmeyer, 2016). Interestingly, the catalytic activity of PARPs is preserved from lower organisms to human beings; ADP-ribosyltransferase enzymes even occur in bacteria and viruses, demonstrating the significance of this modification in evolution.

A key feature of ADP-ribosylation is that it is reversible and also dynamically regulated. Just as kinases add phosphate groups and phosphatases remove them, ADP-ribosylation cycles are maintained by opposing enzymes: ARTs that add ADP-ribose (writers) and hydrolases that remove ADP-ribose (erasers). For MARylation, several ADP-ribosylhydrolases have been identified in recent years, for example, macrodomain-containing proteins like MACROD1/2 which were known to bind MAR and PAR (Karras et al., 2005), were later revealed to detach MAR from aspartate or glutamate, acting as MAR hydrolases (Rosenthal et al., 2013) and enzymes such as ARH3 (Abplanalp et al., 2017; Fontana et al., 2017; Kasamatsu et al., 2011; M. Wang et al., 2018) and TARG1 (Jankevicius et al., 2013), which can specifically hydrolyze mono-ADP-ribose from modified serine/tyrosine (by ARH3), arginine (by ARH1), or other acidic aminoacids (by TARG1). Poly(ADP-ribose) chains, on the other hand, are primarily catabolized by PARG (Poly-ADP-ribose glycohydrolase), which cleaves PAR chains into mono-ADP-ribose units (Slade et al., 2011). Of note, mice deficient of PARG die during embryogenesis resulted by PAR accumulation and cellular apoptosis (Koh et al., 2004). Together, these hydrolases make sure that ADP-ribosylation marks can be removed when signals dissipate, imparting a highly dynamic and reversible character to this modification. This plasticity is biologically important: for instance, in immune cells, the rapid addition and removal of ADP-ribose allows transient activation of pathways in response to stimuli, then timely termination to reset signaling (Rosado & Pioli, 2021). "Writers" (PARPs/ARTs), "erasers" (PARG, ARH family, etc.), and "readers" (binding domains recognizing ADP-ribose, e.g., macrodomains) together constitute a complex regulatory network for ADP-ribosylation, similar to the networks for phosphorylation or ubiquitination (Lüscher et al., 2022; Rosado & Pioli, 2021) (Fig. 7B). Notably, macrodomains within some proteins (including within PARPs themselves or viral proteins) can bind ADP-ribose and modulate signaling by sequestering or removing the modification (Parthasarathy & Fehr, 2022). A good example is Chikungunya virus macrodomain, which can hydrolytically remove ADP-ribose units added onto host proteins by PARP enzymes, thereby suppressing the host's ADP-ribosylation defense mechanisms (Eckei et al., 2017).

While proteins are the most commonly studied substrates for ADP-ribosylation, there is recent evidence to indicate that this modification is not limited to proteins. Recent studies have shown that nucleic acids, DNA and RNA, can be ADP-ribosylated by certain enzymes. For example, PARP1, PARP2, PARP3, and PARP10 have been demonstrated *in vitro* to attach ADP-ribose to DNA or RNA strands (e.g., at strand break ends or specific RNA motifs) (Weixler et al., 2021). A mammalian enzyme, TRPT1 (tRNA 2′-phosphotransferase), initially described in fungal tRNA splicing (Westaway et al., 1988), has the ability to transfer ADP-ribose

onto RNA during tRNA splicing, therefore suggesting an RNA ADP-ribosylation pathway. These nucleic acid ADP-ribosylation reactions are reversible by hydrolases like PARG and ARH3 as well (Munnur et al., 2019; Munnur & Ahel, 2017). Furthermore, ADP-ribose can be conjugated to small metabolites or as intermediates on ubiquitin-like modifiers (as in some specialized pathways), which is indicative of non-canonical ADP-ribosylation beyond standard protein modification. In summary, ADP-ribosylation has the potential to target many numbers of molecules, greatly expanding its potential regulatory range and roles (Lüscher et al., 2022).

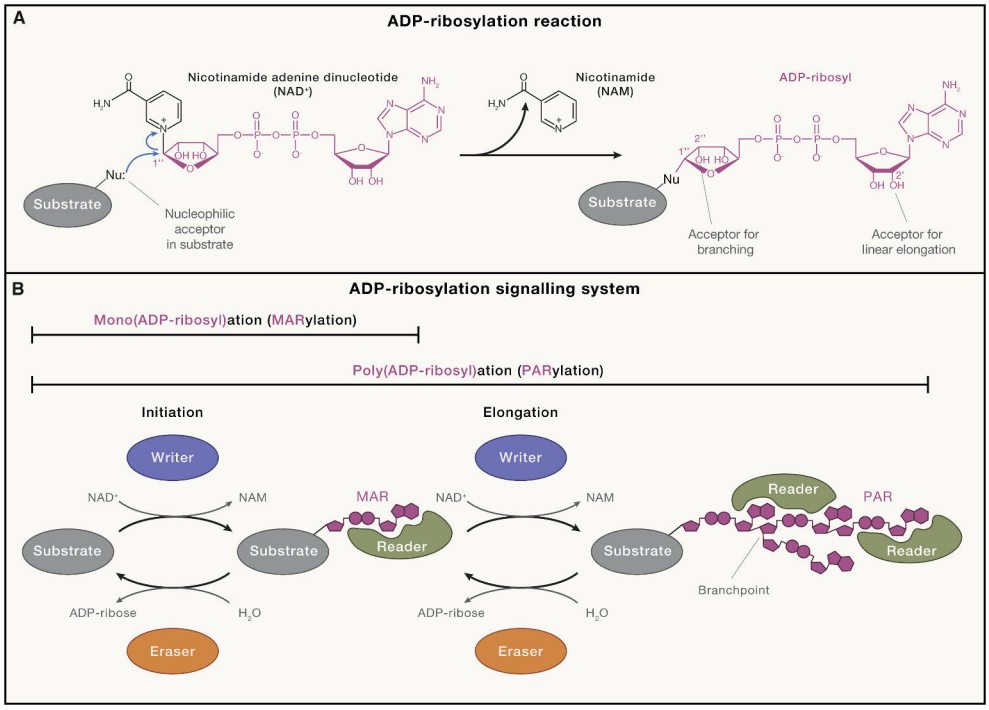


Figure 7. ADP-ribosylation reaction and signalling system. A. Simple illustration of ADP-ribosylation reaction. The blue curly arrow shows nucleophilic attack of an available nucleophilic acceptor functional group (Nu) of the substrate at C1'' of NAD+. The ADP-ribosylation reaction can be repeated after incorporation of the initial ADP-ribose moiety into the acceptor using either 2' or 2'' hydroxyl function of the initial ADP-ribose as nucleophilic acceptor for elongation to give linear or branched chains, respectively. **B.** Regulation of ADP-ribosylation by writers, erasers and readers. Initiation phase has addition of a single ADP-ribose moiety (which is recognized by reader)- constituting MARylation- and followed by elongation (if the writer is a PARylating ART enzyme) with the repetitive rounds generating PARylation. Figure is from Suskiewicz et al., 2023, licensed under CC-BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

In the recent past, the field of ADP-ribosylation has benefited from new tools and high-throughput methods that have made possible better investigation of this

post-translational modification. Biochemical reagents (such as NAD^+ analogs and clickable ADP-ribose probes) and pan-ADP-ribose probes (such as binding domains and specific antibodies) have improved cell-based detection of ADP-ribosylated proteins. Despite these developments, validation has indicated that the detection reagents vary in specificity and sensitivity for some types of ADP-ribosylation. Systematic testing of various ADP-ribose detection methods was conducted by Weixler et al. (2021) and they proved that factors like sample preparation or chemical stability of ADP-ribose linkages have the ability to influence what is being detected (Weixler et al., 2021). Such findings show the importance of using multiple complementary approaches to profile the "ADP-ribosylome." Despite these advances, there remain significant gaps in our knowledge. Many of the 17 PARP family members are still poorly characterized: we know the general enzymatic capability (which are MAR vs PAR producers), but the specific biological processes and substrates for many PARPs are not fully defined. For example, while PARP1 and PARP2 have been known for decades for their roles in DNA repair, and tankyrases (PARP5a/5b) in Wnt signaling and telomere maintenance, other PARPs like PARP8, PARP11, or PARP16 are less clear. As Feijs-Žaja et al. (2024) note, "relatively little is still known about the biological function of most PARPs," which underscores the need for more research (Feijs-Žaja et al., 2024). One of the most recent interests in this field is the efficacy of PARP inhibitors (PARPi) in oncology, PARP1/2-targeting therapies have been identified as potential drugs for BRCA-mutant cancers, and this has established PARPs as drug targets. This has set open avenues to research other members of the PARP family (especially mono-ARTs) as potential therapeutic targets in disorders ranging from cancer to inflammatory disease (Morone & Grimaldi, 2024). In general, ADP-ribosylation is a complex and dynamic system of post-translational modifications. It is written by a family of PARP/ART enzymes, erased by site-specific hydrolases, and read by reader domains. Its roles cover many cellular processes and even host-pathogen relationships.

2.4.2 PARP14 and its Function in Immune Cells

PARP14 is a member of the PARP family that has attracted increasing interest due to its immunomodulatory functions. It is the largest PARP family protein (all mammals-approx. 1800 amino acids) and contains multiple domains: two RNA recognition motifs (RRM), eight K-homology (KH) domains, three tandem macrodomains (inserted into 7th KH domain, separating its N- and C- terminal segments by ~600 residues), a WWE (Trp-Trp-Glu) domain and a C-terminal catalytic domain capable of mono-ADP-ribosylation (Suskiewicz et al., 2023) (Fig. 8). This catalytic domain with PARP homology was isolated and found using *in vitro*

studies to have an auto-MARylation activity (Thorsell et al., 2017). This catalytic domain comprises the H-Y-L motif (instead of H-Y-E (His-Tyr-Glu) motif, that is conserved in most PARPs (Wahlberg et al., 2012)), where the leucine (L) residue replaces the glutamate (E) that is usually required for poly-ADP-ribosylation, and therefore, PARP14 is limited to mono-ADP-ribosyltransferase activity (Kleine et al., 2008).

Figure 8. Domain architecture of human PARP14. PARP14 is made up of RRM (RNA-recognition motif domains), KH (K homology domains), MACRO (macrodomains), WWE (tryptophan-tryptophan-glutamate domain) and ART (ADP-ribosyltransferase domain). The N-terminal half of PARP14 consists of a series of nucleic acid–binding RRM- and KH domains. The seventh of the eight KH domains is split in two by insertion of the three macrodomains. The C terminus consists of a WWE domain followed by the MARylating PARP homology domain (or mART domain) (Suskiewicz et al., 2023). Illustration created with biorender.com.

The following literature review focuses on studies published before 2018 to reflect the scientific context at the outset of the research project. Relevant literature published between 2018 and 2025 is considered and discussed in detail in the 'Discussion' section of this thesis. PARP14 was initially discovered in the context of B cell biology and described as a gene upregulated in certain lymphomas (in B-aggressive lymphoma, hence BAL2) and as a co-activator of STAT6 (hence CoaST6) in IL-4 signaling pathways (Aguiar et al., 2000, 2005; Camicia et al., 2015; Goenka et al., 2007). These early findings hinted at a role in immune cell function and cytokine signaling. It was further found to be necessary for IL-4/IL-13-mediated gene expression in Th2 cells (Krishnamurthy et al., 2017; Mehrotra et al., 2013). When stimulated by IL-4, STAT6 activation induces PARP14's catalytic activity to ADP-ribosylate target proteins in order to regulate chromatin and transcription factor function (Iwata et al., 2016; Krishnamurthy & Kaplan, 2016). One key mechanistic feature is that PARP14 can ribosylate histone deacetylases HDAC2/3 at IL-4-responsive promoters, resulting in their dissociation from chromatin; this relieves repressive transcriptional constraint and facilitates histone acetylation, enabling STAT6 and its co-activators (e.g., p300/CBP) to bind and drive gene transcription (Krishnamurthy & Kaplan, 2016; Mehrotra et al., 2015). PARP14, in other words, acts as STAT6's "transcriptional switch": without cytokine signal, it maintains loci in repressed state, while with IL-4 stimulation, it becomes an active co-activator. PARP14's catalytic (ADP-ribosyl) function is necessary for so doing, since PARP inhibitors pharmacologically mimic PARP14 deficiency by inhibiting IL-4–dependent gene transcription and Th2 cytokine production (Krishnamurthy &

Kaplan, 2016; Mehrotra et al., 2013). PARP14's regulatory effects turn out to be selective for Th2-type genes, enhancing Th2 cytokine genes while inhibiting Th1-type genes in T cells, consistent with its role as STAT6's co-factor and an IFN-γ/STAT1 signaling antagonist (Iwata et al., 2016; Riley et al., 2013). Mechanistically, PARP14 can directly modify STAT1 by mono-ADP-ribosylation, inhibiting STAT1 phosphorylation and downstream pro-inflammatory gene induction (Iwata et al., 2016). PARP14 is thereby an IL-4/STAT6-boosting and IFN-γ/STAT1-restraining STAT co-regulator, skewing immune outputs in a direction toward Type 2 phenotype.

Consistent with its molecular roles, PARP14 is needed in multiple immune subpopulations. In $CD4^+$ T cells, PARP14 is necessary for Th2 differentiation. PARP14-deficient cells have significantly low IL-4, IL-5 and IL-13 production upon Th2 polarization, confirming a role in Th2 lineage specification (Mehrotra et al., 2013). Concurrently, PARP14 loss re-activates Th1-characteristic genes, suggesting movement toward pro-inflammatory (Th1) programming in its absence (Riley et al., 2013). In addition to Th2 cells, PARP14 is equally highly expressed in Th17 cells and induces Th17 differentiation: PARP14 removal or PARP inhibits IL-17A/IL-17F production and Th17 differentiation in vitro and in therapeutic models of allergic airway inflammation (Mehrotra et al., 2015). It also supports T follicular helper (Tfh) cell differentiation, as attested to by decreased frequencies of Tfh cells and germinal center responses in PARP14 deficient mice following immunization (Mehrotra et al., 2015). Further evidence suggests PARP14 activity also supports Th9 polarization (IL-9–secreting cells), indicating its function in yet another T helper subpopulations in addition to Th2 (Riley et al., 2013). In B cells, which use IL-4/STAT6 pathways for processes such as class-switch recombination, PARP14 is also an agonostic regulator. In normal B cells, PARP14 maintains antibody responses: PARP14-deficient mice have decreased IgE in allergic responses and decreased germinal center B cells and antigen-specific antibody titre following immunization (Cho et al., 2011; Mehrotra et al., 2013, 2015). Another key context in which PARP14's immunoregulatory function is needed is in macrophages. In M2-polarized (IL-4–activated) macrophages PARP14 is induced and coordinates alternative activation program (arguably anti-inflammatory), while in M1 (IFN-γ–activated) macrophages acts as inhibitory brake upon pro-inflammatory signaling. PARP14 knockdown/silencing induces macrophage polarization into M1 phenotype by increased STAT1 phosphorylation and pro-inflammatory gene expression, while inhibits contrast-wise IL-4/STAT6–mediated M2 genes (Iwata et al., 2016). Altogether, in immune cells PARP14 operates so as to induce anti-inflammatory or Type 2-biased responses (Th2 cells and M2 macrophages, and related IgE/B cell support) and restrains strongly pro-inflammatory pathways (Th1/IFN-γ axis and M1 macrophages). Such functions position PARP14 as context-dependent regulator of

immunity, and have implications in disease. In models of allergic inflammation e.g., asthma, for instance, PARP14 deletion/knockdown reduces airway inflammation, inhibits eosinophilic infiltration and reduces Th2 cytokines and IgE, suggesting its pathogenic role in allergic asthma (Mehrotra et al., 2013). In atopic dermatitis (skin allergy), PARP14 however is seen to have protective role in some cases: in mice harboring hyperactive STAT6 signaling, PARP14 loss exacerbates dermatitis, suggesting PARP14 operates to inhibit uncontrolled Th2-driven epidermal inflammation (Krishnamurthy et al., 2017). PARP14 has been implicated in host defence against tuberculosis, in one proteome screen PARP14 was among markers of clearance of *Mycobacterium tuberculosis* (*Mtb*), highly suggestive of its participation in macrophage anti-TB response (Kaewseekhao et al., 2015). They found PARP14 peptide to be 1.99-fold downregulated in day 1 -infected vs -uninfected THP-1 cells and 3.21-fold upregulated in day 5 -infected vs -uninfected THP-1 cells through their LC MS/MS analysis of *Mtb* infected THP-1 cells. Based on this difference of day 5 vs day 1 upregulation vs downregulation phenomenon, PARP14 was classified as one among many clearance markers of *Mtb* in human macrophages (Kaewseekhao et al., 2015). They also found PARP14 to be one of the three the clearance markers that overlapped with transcriptional markers identified from TB patients after 26 weeks of treatment with anti-TB drugs (Cliff et al., 2013; Kaewseekhao et al., 2015). Finally, PARP14 overexpression in some lymphomas (e.g., DLBCL) and other cancer subtypes shows its significance as appealing therapeutic target in cancer, in which its pro-survival and metabolic functions (e.g., glycolysis induction and repression of JNK/STAT1-mediated apoptosis) can promote tumor cell growth (Papa & Bubici, 2016). In summary, PARP14 appears to be an immunoregulatory enzyme that modulates cytokine signalling and consequently regulates the balance of inflammation and anti-inflammation responses in various immune cells and disease states.

3 Aims

Gastrointestinal inflammation, as observed in conditions such as inflammatory bowel disease (IBD) and enteric infections, is driven by complex molecular processes. Prior to 2018, poly-(ADP-ribose) polymerase 14 (PARP14), a multidomain mono-ADP-ribosyltransferase enzyme, had been implicated in modulating cytokine signaling in B cells, T cells and macrophages (see section 2.4.2). Despite its recognized roles in immune regulation in other contexts (allergic airway and skin inflammation and eosinophilic esophagitis), the regulation and function of PARP14 in gastrointestinal inflammation remained fully unknown, especially within the framework of established mouse models.

A critical gap existed in our understanding of how PARP14 is expressed, regulated, and functionally engaged during gastrointestinal inflammatory events. In the absence of detailed characterization, the molecular pathways modulated by PARP14 in the gut during inflammation are largely speculative. This knowledge gap hinders our ability to fully appreciate its potential as an immunomodulatory factor, including its potential as a drug target.

The overall aim of this study was to comprehensively characterize PARP14 expression and functions in the context of gastrointestinal inflammation. By integrating analyses of human colon biopsies from IBD patients with studies in murine models (oral dextran sulfate sodium (DSS)-induced colitis and *Salmonella* infection), the study aimed to elucidate the immunomodulatory effects of PARP14 deficiency in the gut.

The specific experimental aims of this Ph.D. thesis research project were:

Aim 1: To characterize PARP14 expression in the intestinal tissues of human IBD vs control patients and of mice from two colitis models (oral DSS exposure, oral *Salmonella* infection).

Aim 2: To characterize macroscopic and microscopic disease symptoms of wt vs. body-wide PARP14 knockout mice in two colitis models (oral DSS exposure, oral *Salmonella* infection) as the proxy of phenotypic PARP14 functions.

Aim 3: To characterize tissue-wide transcriptional patterns of wt vs. body-wide PARP14 knockout mice in two colitis models (oral dextran sulphate sodium exposure, oral *Salmonella* infection) as the proxy of molecular PARP14 functions.

4 Materials and Methods

The experimental procedures described below were performed as reported in Vedantham et al., 2024 (Publication I) and Vedantham et al., 2025 (Publication II).

4.1 Reagents and Antibody Validation

4.1.1 Reagents and Antibodies

A synthetic DNA fragment encoding amino acids 291–358 of human PARP14 (Uniprot Q460N5) was obtained from Eurofins Genomics. This fragment corresponds to the epitope used to generate the mouse monoclonal anti-PARP14 antibody (sc-377150, Santa Cruz Biotechnology). The fragment was cloned into the pGEX-6-P1 vector using BamHI and XhoI restriction sites to produce an N-terminal GST-tagged construct (pGEX-6-P1-PARP14$^{291–358}$). The plasmid was transformed into BL21(DE3) cells. Cultures were grown at 37°C with shaking until the OD 600 reached approximately 0.5, and protein expression was induced with 500 µM isopropyl β-D-1-thiogalactopyranoside. After a 4-hour incubation at room temperature, cells were harvested, lysed in 50 mM Tris (pH 8.0), 150 mM NaCl, and 2 mM DTT (with 0.5 mg/mL lysozyme and protease inhibitors), and the GST–PARP14$^{291–358}$ protein was purified using Protino Glutathione Agarose beads (Macherey-Nagel) according to the manufacturer's instructions.

4.1.2 Antibody Validation and Epitope Competition

For Western blot validation, HEK293T cells were transfected with plasmids encoding full-length human or mouse PARP14. A synthetic DNA fragment (Eurofins Genomics) encoding for the entire mouse Parp14 (Uniprot Q2EMV9) was cloned into pcDNA3.1-Hygro(-)-based human EGFR-HA expression plasmid (Merilahti et al., 2017). The human EGFR insert was replaced with the mouse Parp14 insert before the HA-tag-encoding area using NheI and NotI (C-terminal HA-tag, pcDNA3.1-Hygro(-)-mParp14). The pcDNA3.1-Flag-Parp14 plasmid to express human Parp14 (N-terminal Flag-tag) has previously been described (Barbarulo et al.,

2013). At 24 h post-transfection, cells were washed with ice-cold sterile PBS and lysed in modified RIPA buffer (50 mM Tris pH 7.5, 400 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA), 75 μM Tannic acid (PARG inhibitor, 403040, Sigma Aldrich), 40 μM PJ-34 (PARP inhibitor, J64413, Fischer Scientific), Pierce protease-phosphatase inhibitor (A32961, Thermo Fisher Scientific) and cleared by high-speed centrifugation at 4°C. Protein concentration was measured from the supernatants with a Bradford protein assay. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with the anti-PARP14 antibody (sc-377150, Santa Cruz Biotechnology) diluted in 5% skimmed milk in TBST. In validation experiments, the primary antibody was pre-incubated for 30 minutes at room temperature with GST–PARP14$^{291–358}$ at a concentration 15× higher than that of the antibody. A reduction or loss of the PARP14-specific signal confirmed antibody specificity. Similarly, for immunohistochemistry (IHC), FFPE colon biopsy sections from an ulcerative colitis (UC) patient were processed (see Section 3.2) and epitope competition was performed by pre-incubating the primary antibody with excess GST–PARP14$^{291–358}$.

4.2 *In vitro* Experiments

4.2.1 Cell Culture Conditions

HeLa229 human cervical adenocarcinoma cells (CCL-2.1, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, 21969035) supplemented with 10% fetal bovine serum (FBS; Biowest, S181B-500), 2 mM L-glutamine (Gibco, 25030081), and 25 mM HEPES (Gibco, 15630-056). THP-1 human monocyte/macrophage cells (TIB-202, ATCC) were maintained in RPMI 1640 medium (Lonza) supplemented with 10% FBS. Differentiation of THP-1 cells was induced by treating them with 10 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) for 24 hours.

4.2.2 Cytokine Stimulation

HeLa229 and THP-1 cells were washed with PBS and incubated with media containing 100 U/mL IFN-α (Sigma, SRP4596), 100 U/mL IFN-γ (Thermo Fisher Scientific, PHC4031), or 10 ng/mL TNF-α (R&D Systems, 210-TA) at 37°C with 5% CO_2. Cells were harvested at 2-, 8-, 12-, 24-, and 48-hours post-stimulation, and cell lysates were prepared (as described in section 4.1.2) for subsequent Western blot analysis of PARP14 expression.

4.3 Human Tissue Samples and Immunohistochemistry

4.3.1 Tissue Procurement and Processing

FFPE colon biopsy specimens (5 µm sections) from anonymous patients diagnosed with ulcerative colitis (UC, n = 12), Crohn's disease (CD, n = 7), and from patients with histologically normal colons (n = 9) were obtained from the Finnish biobank system (https://site.fingenious.fi/en/, Auria Biobank, Turku, Finland).

4.3.2 Immunohistochemistry (IHC) for Human Tissues

Tissue sections were air-dried for 2 hours at room temperature and then incubated overnight at 37°C. Sections were deparaffinized in xylene and rehydrated through a graded alcohol series. Endogenous peroxidase activity was blocked using a Peroxidase blocking solution (Abcam, ab64259). Antigen retrieval was performed by immersing sections in prewarmed 10 mM sodium citrate buffer (pH 6.0) and boiling in a water bath for 20 minutes. Following rinsing in PBST (Phosphate Buffered Saline with 0.01% Tween-20), sections were blocked in 5% BSA (w/v) in PBST for 1 hour at room temperature. The primary anti-PARP14 antibody was diluted in 5% BSA and applied overnight at 4°C. For epitope competition controls, the primary antibody was pre-incubated with excess GST-PARP14$^{291–358}$. After washing, sections were incubated with a biotinylated anti-mouse secondary antibody (provided with the IHC kit) for 1 hour at room temperature, followed by incubation with streptavidin-HRP and development using DAB as the chromogen. Counterstaining was performed with Harris hematoxylin, and sections were mounted in Histo-Clear. Two persons, one being a pathologist, independently and blind for the sample group, scored the stained sections (1:10 000 of anti-PARP14 antibody) by visual inspection using a bright field microscope. The PARP14 staining intensity (0-none, 1-faint and irregular, 2-mild and regular, 3-strong and highly regular) was scored for the surface epithelial cells and the cryptal epithelial cells. The score of one particular patient means the average of scores from all the available tissue sections across the distal gastrointestinal tract (terminal ileum, ileocecal valve, cecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum), which varied from patient to patient. The scores of the two persons were averaged. Differences between cryptal and surface epithelial cell staining intensities in patient groups were summarized with descriptive statistics and studied by the Kruskal-Wallis test. The normality of variables was evaluated visually and tested with the Shapiro-Wilk test. Due to the non-normality of the continuous variables, nonparametric methods were used. The statistical significance level was set at .05 in

all tests (two-tailed). The analyses were performed using the SAS system, version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA).

4.3.3 Immunohistochemistry and Immunofluorescence of Murine Tissues

Murine intestinal tissues were processed for IHC and immunofluorescence using procedures similar to those for human tissues. FFPE sections were deparaffinized, rehydrated, and subjected to antigen retrieval in prewarmed 10 mM sodium citrate buffer (pH 6.0) by boiling for 20 minutes. For IHC, sections were blocked in 5% BSA and incubated overnight at 4°C with the anti-PARP14 antibody (dilution 1:500). Detection was performed using a biotinylated secondary antibody, streptavidin-HRP, and DAB, with counterstaining by Harris hematoxylin. For immunofluorescence, after blocking, sections were incubated overnight at 4°C with a cocktail of primary antibodies: anti-PARP14 (sc-377150, 1:500) and a rat anti-mouse F4/80 antibody (Alexa Fluor 647-conjugated, MCA497A647, Biorad, 1:500). After washing, sections were incubated with appropriate secondary antibody (e.g., Alexa Fluor 488-conjugated goat anti-mouse IgG for PARP14) and counterstained with DAPI. Slides were mounted in an antifade mounting medium and imaged using a Zeiss AxioImager M1 microscope.

The anti-Parp14 stained IHC tissue sections of wt mice (distal small intestine, cecum, and proximal large intestine) were scanned using a Pannoramic 1000 Slide scanner (3DHistech, Budapest, Hungary) with 40x objective and analyzed with a Pannoramic viewer (3D Histech, software version 1.15.4). Scanned slides were converted to .mrxs file type and specific project in QuPath (qupath.github.io, software version 0.5.1, (Bankhead P et al., 2017)) was created to quantify the DAB OD (optical density). Parameters for stain vectors of Hematoxylin and DAB were set using automatic estimation in QuPath. For cellular detection, nucleus parameters used were: background radius - 8µm, minimum area −10µm^2 and maximum area - 400µm^2. Cell expansion was set at 5µm. From each tissue of each section, minimum 50 to maximum 200 horizontal villus crypts were selected for cellular detection and quantifying DAB staining intensity. Detected annotations were exported and statistical analyses were conducted using the one-way ANOVA with Tukey's multiple comparison test to compare the means.

4.4 Animal Models

4.4.1 Mouse Strains and Genotyping

Wild-type and body-wide PARP14 knockout mice were maintained under specific-pathogen-free conditions. The PARP14 knockout mice were generated as described in Cho et al., 2009 and backcrossed for 10 generations to the C57BL/6N background (used for DSS colitis and *Salmonella* infection experiments). Genomic DNA was extracted from ear biopsies of 2-3 week old mice. PCR-based genotyping was performed using the following primer sets:

- Wild-type: 5′-GGCCTAACTATTCACTCGTGT-3′ and 5′-CTGCTCTTCTAGATGATGCAGA-3′
- Knockout: 5′-GATGCAACTGCAAGAGGGTTTAT-3′ and 5′-CTGCTCTTCTAGATGATGCAGA-3′.

4.4.2 *Salmonella* Infection Model

Female C57BL/6N mice (6-8 weeks old) were used. Mice were fasted for 4 hours and then administered 20 mg of streptomycin (75 µL sterile solution) by oral gavage. Twenty hours later, following an additional 4-hour fast, mice were orally gavaged with 50 µL of PBS containing 10^8 colony-forming units (CFU) of *Salmonella enterica* serovar Typhimurium (SL1344) or with 50 µL PBS as a control. Post-infection, mice were provided drinking water ad libitum, with food restored 2 hours later. Mice were euthanized by CO_2 asphyxiation at specified time points (e.g., day 1 and day 5 post-infection). Organs (liver, spleen, MLN, distal small intestine, proximal colon, and cecum) were collected, weighed, and processed for bacterial viability determination (Section 4.4.3), histology (fixed in 4% paraformaldehyde), or RNA analysis (snap-frozen in liquid nitrogen) (Fig. 1B, Publication II).

4.4.3 Bacterial Viability Determination

Collected tissues were homogenized in ice-cold PBS using stainless steel balls and a bead mill. Serial dilutions of the homogenates were plated on Luria-Bertani (LB) agar containing streptomycin (50 µg/mL). Plates were incubated at 37°C for approximately 12 hours, after which CFUs were counted. Bacterial load was expressed as CFU per gram of tissue. Statistical analyses were performed as mentioned in section 4.9. The statistical significance level was set at .05 in all tests (two-tailed).

4.4.4 DSS Colitis Model

Male C57BL/6N mice (6–8 weeks old) were randomly assigned to control or DSS treatment groups. Mice were housed in standard groups of 2 animals per cage during the experiment. Clinical colitis severity scoring system was adapted from Cooper et al., 1993, which has been widely validated in DSS colitis models. The DSS-treated group received drinking water containing 2.5% (w/v) dextran sulfate sodium (DSS; molecular weight 40,000 Da; TdB Consultancy AB, Uppsala, Sweden) for 7 days. Mice were weighed daily, and stool consistency and rectal bleeding were recorded using the following scoring criteria:

- Blood in stool: 0 = none; 1 = small amounts; 2 = blood throughout the pellet; 3 = clotted blood at the anus; 4 = fresh blood on the mouse or bedding.
- Stool consistency: 1 = normal; 2 = formed but soft; 3 = slightly loose; 4 = liquid.

For analyses, weight was converted to percentages. Statistical analyses were performed as mentioned in section 4.9. The statistical significance level was set at .05 in all tests (two-tailed).

After 7 days of DSS treatment, mice were euthanized by CO_2 asphyxiation. The colon was excised, measured for length (in mm), and divided into proximal, mid, and distal segments. Blood was collected via cardiac puncture, and spleens were harvested. Tissue samples for RNA analysis were snap-frozen in liquid nitrogen, and samples for histology were fixed in 4% paraformaldehyde.

4.5 Histopathological Analysis

4.5.1 Tissue Processing and H&E Staining

Tissues fixed in 4% paraformaldehyde were processed routinely and embedded in paraffin. Sections (5 µm thick) were cut and stained with hematoxylin and eosin (H&E) using standard protocols. Slides were scanned using a Pannoramic 1000 Slide Scanner (3DHistech) at 20× magnification.

4.5.2 Histopathological Scoring

Histopathological scoring criteria were adapted from Dieleman et al., 2001. H&E-stained sections were evaluated by two independent and blinded observers. The following parameters were scored:

- Immune cell infiltration (0–3)
- Edema (0–3)
- Epithelial erosion (0–3)
- Goblet cell loss (0–3)

Scores for each parameter were recorded by two people in a double blinded manner and averaged for each sample. Statistical analyses were performed as mentioned in section 4.9. The statistical significance level was set at .05 in all tests (two-tailed).

4.6 Flow Cytometry

4.6.1 Preparation of Single-Cell Suspensions

For colon samples, the mid-colon was excised, and surrounding fat was removed. The colon was opened longitudinally, rinsed in PBS to remove fecal material, and cut into 5–10 mm pieces. Tissue pieces were incubated in 5 mL prewarmed 2 mM EDTA in Hank's balanced salt solution (HBSS) at 37°C with shaking (250 rpm) for 15 minutes to remove epithelial cells. Residual tissue was subjected to additional EDTA treatments if necessary. The remaining lamina propria was digested in 5 mL of enzyme cocktail containing 1 mg/mL Collagenase VIII (Sigma, C2139) and 10 µg/mL DNase I (Sigma, D5025) in RPMI supplemented with 1% FCS at 37°C with shaking (250 rpm) for 45 minutes. The reaction was stopped by adding 5 mL cold FACS buffer (PBS with 2% FCS and 1 mM EDTA), and the suspension was filtered through a 70 µm cell strainer.

For blood, approximately 100 µL was collected into heparinized tubes, and red blood cells were lysed by treatment with 0.2% NaCl followed by restoration with 1.6% NaCl. Spleens were mechanically dissociated through a metal mesh, and red blood cells were lysed using the same hypotonic treatment.

4.6.2 Immunostaining and Data Acquisition

Cells were first stained with Fixable Viability Dye eFluor 780 (eBioscience) to exclude dead cells. Fc receptors were blocked by incubating cells with unconjugated CD16/32 antibody (BioXCell, clone 2.4G2). Cells were then stained for 30 minutes at 4°C with fluorophore-conjugated antibodies against markers including CD45, CD4, CD8, B220, CD11b, CD11c, Ly6C, Ly6G, and F4/80. After staining, cells were washed with FACS buffer and fixed in 1% formaldehyde. Data were acquired on an LSRFortessa flow cytometer (BD) using FACSDiVa software and analyzed with FlowJo.

4.7 Fecal Microbiota Analysis

4.7.1 DNA Extraction and 16S rRNA Gene Amplification

Fecal pellets were processed using the NucleoSpin DNA Stool kit (Macherey-Nagel) according to the manufacturer's protocol. The nearly full-length 16S rRNA gene was amplified using barcoded primers (forward: AGRGTTYGATYMTGGCTCAG; reverse: RGYTACCTTGTTACGACTT) and the 2× Roche KAPA HiFi Hot Start Ready Mix.

4.7.2 Sequencing and Bioinformatic Processing

Amplicon libraries were prepared using the SMRTBell Express Template Prep kit 2.0 (PacBio) and sequenced on a PacBio Sequel IIe platform using circular consensus sequencing (CCS) mode with a 12-hour movie time. Sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold using mothur (v1.48.0). Alpha-diversity (Chao1 and Shannon indices) and beta-diversity analyses were performed using R software.

4.8 RNA Isolation and Transcriptomic Analysis

4.8.1 RNA Isolation

Colon tissues were homogenized in TRIsure reagent (BIO-38033, Bioline GmbH) using stainless steel balls and a TissueLyser LT (Qiagen). After homogenization, chloroform was added for phase separation, and RNA was precipitated using isopropyl alcohol. The RNA pellet was washed with 70% ethanol, dissolved in nuclease-free water, and treated with RNase-free DNase (Macherey-Nagel) according to the manufacturer's instructions. RNA concentration and purity were measured using a DeNovix DS-11 spectrophotometer. The experimental steps as described in 4.8.2. and 4.8.3. were performed by Novogene Co., Ltd (Cambridge, UK, https://www.novogene.com/eu-en/).

4.8.2 Library Preparation and Sequencing

Messenger RNA was enriched using poly-T oligo-attached magnetic beads. Fragmentation was performed with divalent cations under elevated temperature in First Strand Synthesis Reaction Buffer (5×). First strand cDNA synthesis was carried out using random hexamer primers and M-MuLV Reverse Transcriptase. Second strand synthesis was then performed using DNA Polymerase I and RNase H,

followed by end repair and 3' adenylation. Adaptors with a hairpin loop structure were ligated to the cDNA fragments, and fragments of preferentially 370–420 bp were purified using the AMPure XP system (Beckman Coulter). PCR amplification was performed using Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. Libraries were purified, quality-assessed on an Agilent Bioanalyzer 2100, and sequenced on an Illumina Novaseq platform to generate 150 bp paired-end reads.

4.8.3 Bioinformatic Analysis

Raw fastq files were processed using in-house perl scripts to remove adapter sequences, poly-N reads, and low-quality sequences. Clean reads were aligned to the reference genome using Hisat2 (v2.0.5). Gene counts were generated using featureCounts (v1.5.0-p3) and normalized to FPKM. Differential expression analysis was performed using the DESeq2 R package, with a $\log_2$ fold-change threshold of ±1 and adjusted p-values <0.05. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted using online tools as described in the original publications.

4.8.4 Quantitative PCR (qPCR) Analysis

For qPCR, total RNA (as isolated in Section 4.8.1) was reverse transcribed using SuperScript III reverse transcriptase (Thermo Fisher Scientific, 1808044) and Oligo (dT) 12–18 Primer (Thermo Fisher Scientific, 18418012). Real-time PCR was performed in duplicates using TaqMan gene expression assays (Assay IDs: Mm00520984_m1 for PARP14; Mm00434228_m1 for IL-1β; Mm00446190_m1 for IL-6; Mm00441242_m1 for CCL2; Mm00443258_m1 for TNF-α; and Mm99999915_g1 for GAPDH) on a Rotor-Gene Q real-time PCR cycler (Qiagen). The thermal cycling conditions were an initial denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Samples were included only if duplicate Ct values had a standard deviation below 0.5. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method with the mean ΔCq-value of day 1 *Salmonella*-infected wild-type mice as the calibrator.

4.8.5 Analysis of Publicly Available Microarray Datasets

Publicly available microarray datasets (accession numbers E-GEOD-14580 and E-GEOD-4183) were obtained from the ArrayExpress database (https://www.ebi.ac.uk/biostudies/arrayexpress). Using the biogps.org gene annotation portal, PARP14 mRNA expression values (fluorescence intensities) were

downloaded for each patient sample. Sample information provided by ArrayExpress was used to assign subjects into IBD (ulcerative colitis and/or Crohn's disease) and control groups. Mean expression values for PARP14 were calculated for each group, and statistical comparisons were performed using the Mann–Whitney test.

4.8.6 Single-Cell RNA-Seq Data Analysis

Published single-cell RNA-Seq data from epithelial-enriched cell suspensions of *Salmonella*-infected mice (Haber et al., 2017) were obtained from the Broad Institute Single Cell Portal (https://singlecell.broadinstitute.org/single_cell). Data were analyzed using the portal's online interface to assess the expression levels of PARP14 across distinct epithelial subpopulations.

4.9 Statistical Analysis

Data were assessed for normality visually and using the Shapiro-Wilk test. For normally distributed data, t-tests or ANOVA were used; for non-normally distributed data, nonparametric tests (Kruskal-Wallis, Wilcoxon signed rank, or Mann-Whitney tests) were applied. Statistical analyses were performed using SAS (version 9.4 for Windows), GraphPad Prism, and R software. A p-value of <0.05 was considered statistically significant.

5 Results

5.1 Human PARP14 Localizes in Colonic Crypt Epithelial Cells

5.1.1 Antibody Validation Confirms PARP14-Specific Detection

Using epitope competition assays, we verified that the commercial monoclonal anti-PARP14 antibody (sc-377150, Santa Cruz Biotechnology) specifically recognizes PARP14. Western blot analyses of HEK293T cell lysates overexpressing full-length human and mouse PARP14 demonstrated that pre-incubation of the antibody with a recombinant GST–PARP14 fragment (amino acids 291–358) resulted in the absence of the specific PARP14 signal (I, Fig. S1). Similarly, epitope competition IHC on an ulcerative colitis (UC) patient biopsy markedly reduced the PARP14 staining intensity (I, Fig. S1).

5.1.2 Differential PARP14 Expression in IBD Patients (I)

5.1.2.1 *In Situ* Evidence from IBD Patient Biopsies

Immunohistochemical analysis was conducted on FFPE colon biopsy sections obtained from patients with ulcerative colitis (n = 12), Crohn's disease (n = 7), and from patients with histologically normal colons (n = 9). In these sections, PARP14 staining was predominantly observed to be localized in the epithelial compartments of the colon. The staining pattern was distinctly observed to be granular and primarily cytoplasmic in the surface epithelial cells (I, Fig. 1C and Fig. S2). PARP14 staining intensity was scored as mentioned in section 4.3.2. Briefly, a pathologist and I, visually scored the PARP14 staining intensity (0, faint to 3, high intensity) in surface (facing the gut lumen) and crypt (facing the lamina propria) epithelial cells by using 1:10000 dilution PARP14 IHC stained tissue sections. Our scores were averaged, and statistical analyses were performed. There was a trend toward less pronounced PARP14 staining in the surface epithelial cells of CD patients as

compared to non-IBD patients (*p*-value .0851). However, we did not find any significant differences between staining intensity of non-IBD vs CD and UC patients. Interestingly, surface epithelial cells showed consistent stronger staining intensity when compared to crypt epithelial cells, in all three groups of patients (I, Fig. 1D). In several UC cases, regions of disrupted epithelial architecture, particularly those with goblet cell loss and local immune cell infiltration, these demonstrated persistent and high PARP14 expression (these were non-quantifiable) (I, Fig. 1B).

5.1.2.2 External Validation of Elevated PARP14 Transcript Levels in IBD

Analysis of publicly available previously published microarray datasets from two independent IBD cohorts: E-GEOD-14580, control, n=6 and UC, n=24, (Arijs et al., 2009) and E-GEOD-4183, control, n=8 and CD & UC, n=15 (Galamb et al., 2008), revealed that PARP14 transcript levels (mean fluorescence intensity) are significantly elevated in colon biopsies from IBD patients compared to the controls (I, Fig. S11).

5.1.2.3 *In vitro* Human Cell Models

To support the previous findings, *in vitro* experiments to study PARP14 protein expression were performed using HeLa229 cells as a human epithelial model and THP-1 cells as a human macrophage model. Under basal conditions, HeLa229 cells displayed minimal levels of PARP14; however, upon stimulation with inflammatory cytokines such as IFN-α and IFN-γ, PARP14 expression was markedly induced (I, Fig. S3). Similarly, THP-1 cells showed a time-dependent increase in PARP14 expression following cytokine treatment (I, Fig. S3). Taken together, PARP14 is expressed in the human colon, in particular by the epithelial cells, with a characteristic granular staining pattern in the cytosol.

5.2 Differential PARP14 Expression in Gastrointestinal Inflammation Disease Models

5.2.1 PARP14 is differentially and dynamically expressed upon inflammation and infection (I & II)

Murine PARP14 expression was evaluated in gastrointestinal tissues from controls (water and PBS-treated) and diseased mice in both, DSS colitis models (FVB/n and C57BL/6N) and *Salmonella* infection (C57BL/6N). In wild-type control mice,

PARP14 staining was generally low, whereas in disease states more pronounced staining was observed in the colon tissues (I, Fig. 2A and B and II, Fig. S1). The staining appeared to be granular in nature in murine tissues too, similar to human IHC staining (I, Fig. S5 and S2).

DSS colitis model: Murine colon tissues from DSS-treated mice were analyzed in two different genetic backgrounds (FVB/n and C57BL/6N). In FVB/n mice, DSS exposure showed strong PARP14 staining in colon crypt epithelial cells compared to water-treated controls. In contrast, in C57BL/6N mice the staining was less pronounced (I, Fig. 2A). Complementary qPCR analysis of parp14 mRNA levels in the DSS colitis model confirmed that in C57BL/6N mice, parp14 transcript levels were significantly higher in the distal colon compared both to water-treated controls and to proximal regions, which was not the case with FVB/n mice, highlighting the strain- and region-specific induction (I, Fig. 2C).

Salmonella infection model: FFPE sections from the small intestine, cecum, and large intestine were stained for PARP14 (II, Fig. S1). Quantitative image analysis using QuPath revealed that in the small intestine, PARP14 staining increased markedly at day 1 post-infection and then declined by day 5. In the cecum, staining intensity decreased following infection, whereas in the large intestine an early increase in staining was observed at day 1 that persisted or further increased by day 5. Further, a dynamic shift in subcellular localization was observed in wild-type mice's large intestine, i.e., PARP14 initially (day 1 p.i.) exhibited predominantly higher nuclear localization, which transitioned to a more pronounced cytoplasmic pattern as infection progressed (day 5 p.i.) (II, Fig. 2A). In addition, qPCR analysis of parp14 mRNA levels was performed on the same tissues in the Salmonellosis model. Here, parp14 transcript levels did not show statistically significant differences between day 1 and day 5 post-infection in the large intestine, small intestine, or cecum (I, Fig. 2D and II, Fig. 2B). It is to be noted that the number of PBS-treated control mice were not enough to perform statistical analysis between control and infected samples for qPCR.

5.2.2 PARP14 co-localizes in intestinal macrophages (I and II)

Double immunofluorescence staining was performed on murine tissues from both disease models using antibodies against PARP14 and the macrophage marker F4/80. Co-localization was observed, indicating that PARP14 is expressed not only in epithelial cells but also in certain macrophage populations (I, Fig. S6 and II, Fig. S2). These combined qPCR, and IHC analyses, quantified using QuPath, demonstrate regional, temporal, and strain-dependent differences in the PARP14 expression in murine gastrointestinal tissues under inflammatory conditions.

5.2.3 Single-Cell RNA-Seq Analysis of Epithelial Subpopulations in the *Salmonella* Model (II)

Analysis of published single-cell RNA-Seq data from epithelial-enriched cell suspensions of *Salmonella*-infected mice (Haber et al., 2017) (sourced from the Broad Institute Single Cell Portal) demonstrated that PARP14 is most prominently expressed in specific epithelial subpopulations, particularly in enterocytes and tuft cells (II, Fig. S4). This cell type–specific expression further supports the central role of PARP14 in modulating epithelial responses during *Salmonella* infection.

5.3 Loss of PARP14 Exacerbates Gastrointestinal Injury and Alters Disease Trajectories

5.3.1 DSS Colitis: Heightened Inflammatory Damage and Impaired Mucosal Repair (I)

5.3.1.1 Clinical Deterioration Reflects Increased Mucosal Vulnerability

In the DSS colitis model, male mice received 2.5% DSS in the drinking water for 7 days. Both wild-type and PARP14-deficient mice showed, to similar extent, weight loss over the treatment period (I, Fig. 3B) and a reduction in colon length on day 8 after ending the DSS-treatment on 7th day (I, Fig. 3E). We also quantified stool consistency (diarrhea) and rectal bleeding, during the entire course of the DSS-treatment. There was no significant difference in stool consistency (I, Fig. 3C), however, the PARP14-deficient mice displayed significantly increased rectal bleeding, when compared to the wild-type mice (I, Fig. 3D).

5.3.1.2 Histopathological Analysis Confirms Exacerbated Colonic Injury

Following the DSS treatment of 7 days, histopathological evaluation of proximal and distal colon sections from day 8 was done by visually quantifying epithelial erosion, goblet cell loss, edema and immune cell infiltration, as described in section 4.5.2. Statistical analysis revealed that PARP14-deficient mice had significantly more severe epithelial erosion, increased goblet cell loss, and heightened immune cell infiltration compared to wild-type controls. Edema scoring showed no significant changes. These differences were consistently observed in the distal colon and were confirmed by independent scoring by 2-person blinded quantification (I, Fig. 3F and G). Higher goblet cell loss was found to be significant also in the proximal colon of PARP14-deficient mice when compared to wild-type mice. This data indicates that the absence of PARP14 is associated with exacerbated inflammatory injury and an impaired reparative response.

5.3.2 *Salmonella* Infection: Increased Mucosal Damage (II)

5.3.2.1 Altered Clinical and Macroscopic Outcomes

In streptomycin-pretreated *Salmonella* infection model, PARP14-deficient and wild-type mice were monitored for weight loss during the course of 5 days of infection. There was no significant weight loss observed between genotypes (II, Fig. 1C). Colon length, spleen and liver weights also showed no significant changes (II, Fig. 1D, E and F). Quantification of *Salmonella* CFUs revealed two significant differences: wild-type mice exhibited higher bacterial burden in the liver at day 1 (II, Fig. 1L), whereas knockout mice showed higher bacterial burden in the colon at day 5 (II, Fig. 1H). No significant genotype differences were observed in the small intestine, feces, spleen, or mesenteric lymph nodes. These findings indicate that PARP14 deficiency may affect bacterial clearance in a tissue- and time-specific manner, although no consistent systemic pattern was observed.

5.3.2.2 Histopathological Evidence of Exacerbated Damage

Distal small intestine, cecum and proximal large intestine (colon) were histologically examined using H&E staining for infection-induced tissue damage. Histopathological changes were quantified as mentioned in section 4.5.2. Statistical analysis revealed *Salmonella*-infected PARP14-deficient mice had an intense inflammatory reaction when compared to wild-type infected mice. On day 1 p.i., of all three regions, only distal small intestine showed significantly higher epithelial erosion in KO-infected mice when compared to WT-infected mice. By day 5, distal small intestine showed no significant histopathological change in the quantified variables. However, by day 5, colon showed increased epithelial erosion, immune cell infiltration, along with extensive loss of goblet cells in KO-infected mice when compared to WT-infected mice. There was no change in edema observed. Cecum had severe inflammatory response upon infection in both genotypes but there was no significant change between KO- and WT-infected mice on both the timepoints (II, Fig. 3A and B).

5.4 PARP14 Modulates Systemic Immune Responses Without Disrupting Gut Microbiota

5.4.1 Altered Peripheral Immune Cell Dynamics in PARP14 Deficiency (I)

Mid-colon, blood, and whole spleen single-cell suspensions of PARP14-deficient and wild-type mice, DSS-treated and water controls, were examined for leukocyte

subclass population analysis using flow cytometry, as described in section 4.6. DSS treatment has a significant effect on mice colon, where percentage of neutrophils and monocytes are high when compared to water control mice. However, all analyzed immune cell numbers in colon tissues (neutrophils, eosinophils, B cells, CD8 T cells, CD4 T cells, F4/80 macrophages and Ly6C low and hi-monocytes) were not significantly different across genotype, under DSS-treatment (I, Fig. 5). However, peripheral compartments showed significant differences. In peripheral blood of mice treated with DSS, classical Ly6C^{hi} monocytes were significantly decreased in PARP14-deficient animals relative to wild-type animals ($p = 0.0322$). Moreover, percentage increase in wild-type animals' CD8 T cells due to DSS was significant ($p = 0.0430$), while this was not seen in PARP14-deficient animals. Further, increase in percentage of circulating CD4 T cells in PARP14-deficient mice after DSS administration ($p = 0.0242$) was not seen in wild-type animals (I, Fig. S8B). In whole spleen tissues, PARP14-deficient mice were found to have proportionately reduced CD8 T cells relative to water-treated controls ($p = 0.0205$), while minimal difference in numbers of CD8 T cells was observed in wild-type animals under similar conditions (I, Fig. S8C). Taken together, these observations verify that, while DSS treatment in colon induces significant neutrophil and monocyte population changes when compared to water controls, changes between genotypes show no statistical change, but PARP14 deficiency causes alterations in peripheral immune cells, namely in distribution among subpopulations of T cells and among monocytes.

5.4.2 Intact Fecal Microbiota in the Absence of PARP14 (I)

Fecal microbiota of the resting wild-type and PARP14-deficient mice were studied by DNA extraction from fecal pellets. Using PacBio long-read sequencing of the nearly complete 16S rRNA genes were amplified using barcoded primers, and amplicon libraries were prepared. We grouped sequences into operational taxonomic units (OTUs) whenever they were at least 97% similar, then identified each OTU's likely bacterial classification (I, Fig. 4A). At all taxonomic levels, from phylum down to species, the total number of different groups found was about the same in wild-type and PARP14-deficient mice. We also plotted the relative abundance of the forty most common genera and species in heatmaps (I, Fig. 4B). Next, we compared within-sample diversity using two standard metrics, Chao1 (richness) and Shannon (richness plus evenness). Neither showed any significant difference between the two mouse strains (I, Fig. 4C), meaning they hosted similar numbers and distributions of bacterial OTUs. Finally, we tested overall community differences (beta diversity) by running principal coordinate analysis (I, Fig. 4D), plus permutational ANOVA and dispersion tests (data not shown). None of these detected a statistically significant shift in bacterial composition between wild-type and PARP14-deficient mice. In

summary, during in-house maintenance, loss of PARP14 did not trigger detectable changes in the colon's bacterial community and revealing that differences in colitis severity in experiments are not due to fecal microbiota.

5.5 Transcriptomic Reprogramming Reveals Mechanistic Insights into PARP14's Protective Role

5.5.1 Dysregulated Inflammatory and Repair Responses in the DSS Colitis Model (I)

RNA-sequencing profiling of the distal-colon tissues of mice subjected to 7-day treatment with DSS and samples taken on the following day, revealed that the transcriptional response of the wild-type mice upon treatment with DSS was characterized by upregulation of cell cycle-progressive genes as well as genes related to DNA replication, reflecting active repair of the tissues. In stark contrast, the transcriptome of the PARP14-deficient mice subjected to treatment with DSS was dominated by inflammatory pathway-related genes as well as infection-related genes. GO (I, Fig. 6A-E) as well as KEGG pathway (I, Fig. 6F-I) enrichment analysis of differentially expressed genes with the selection parameters of $\log_2$ fold-change ≥ 1 or ≤ -1 and the adjusted p-values ≤ 0.05 identified wild-type mice to be characterized by high levels of significant enrichment of pathways related to regeneration of tissues (I, Fig. 6 B and F). In comparison, PARP14-deficient mice were dominated by high levels of expression of inflammatory mediators with relatively few genes related to proliferation as well as response to repair (I, Fig. 6 C and G). The pattern is consistent with histopathological scoring observed with PARP14-deficient mice and is molecular evidence of defective mechanisms of recovery observed with the mice.

5.5.2 Basal Transcriptomic Dysregulation Predisposes to Inflammation (I)

In order to assess whether PARP14 deficiency affects the resting state of the colon transcriptome, RNA-Seq analysis was performed on colon tissues from water-treated (control) mice. We were interested in two types of data: numbers of genes detected to be expressed (FPKM > 1), as well as the differential gene expression (DEG) data (I, Fig. S9 D and E). The DEG data analysis identified a set of genes that were significantly upregulated (n= 106) and downregulated (n= 78) in PARP14-deficient mice compared to wild-type mice (I, Fig. 7A and B). The upregulated genes' enrichment analysis did not reveal any GO terms. GO enrichment analysis of the

downregulated genes showed a strong association with biological processes (BPs, n=11) related to the regulation of inflammation and microbial defense. KEGG pathway analysis further indicated that inflammation and infected related cellular functions were suppressed; however direct functional downregulation was not assessed (I, Fig. 7 D and E).

Additionally, we analyzed genes that were detected to be expressed in only water-treated wild-type mice (where mean FPKM >1) and not at all in PARP14-deficient mice (mean FPKM <1). Based on KEGG analysis, we found that 51 out of 639 such wt genes were involved in immune regulation and mucosal homeostasis, i.e., these were absent in the knockout mice (I, Fig. S10). These basal transcriptional differences suggest that even in the absence of an external inflammatory stimulus, the colon of PARP14-deficient mice exhibits a predisposition to an altered and probably weaker inflammatory state, potentially contributing to the increased rectal bleeding and severe tissue damage observed following DSS treatment.

5.5.3 Dysregulated Inflammatory and Cellular Dynamics in the *Salmonella* Model (II)

Bulk tissue RNA sequencing was carried out on distal colon samples from *Salmonella*-infected mice (day 1 p.i.). Large number of genes were detected in both wild-type and PARP14-deficient groups using a standard FPKM cutoff (FPKM >1). In addition, subsets of genes were identified as being uniquely expressed in each genotype, with approximately 520 genes detected to be exclusive in wild-type animals and 325 genes unique to the knockout mice (II, Fig. 4A). GO analysis of the wild-type–specific gene set revealed a significant enrichment in BPs associated with immune responses (7 out 23 identified BPs), including pathways such as neutrophil chemotaxis, leukocyte migration, which included cytokines such as *ccl17*, *ccl7*, *ccl2*, *cxcl9* and *cxcl10*. KEGG pathway analysis revealed IL-17 signaling cascade (consisting of 8 genes, *lcn2*, *il1b*, *s100a8*, *s100a9*, *cxcl10*, *ccl17*, *ccl7*, *ccl2*), unique to wt gene set. In contrast, the genes uniquely expressed in PARP14-deficient mice were enriched for functions related to cell division and cytoskeletal organization (II, Fig. 4 B, C and D).

Differentially expressed genes (DEGs) analysis (using thresholds of $\log_2$ fold-change >1 or <–1 and adjusted p-values <0.05) using GO and KEGG enrichment analysis showed that many genes involved in cell adhesion and the maintenance of epithelial integrity were downregulated in the knockout mice (II, Fig. 6C, D and E). These data collectively provide a detailed transcriptional landscape that distinguishes the response of wild-type animals, characterized by a coordinated activation of immune signaling, from that of PARP14-deficient mice, in which processes vital to epithelial repair appear to be compromised.

5.5.4 Cytokine Responses in Distinct Intestinal Regions during *Salmonella* Infection (II)

To corroborate and complement RNA-Seq data (from day 1 p.i.), TaqMan qPCR experiments were conducted using proximal large intestine, cecum, and distal small intestine RNA from infected mice at day 1 and day 5 post-infection. Day 1 following infection in large intestine showed that expression of IL-1β, CCL2, and IL-6 were all significantly higher in wild-type mice compared to PARP14-deficient mice. Day 5 following infection, however, saw differences in large intestine cytokine expression drop below statistical significance (II, Fig. 5). According to RNA-seq data, IL-1β and CCL2 were found to be unique for wild-type infected mice, i.e., these were among the 520 genes that were not detected in PARP14-deficient infected mice, as mentioned in the previous section. We showed using qPCR of the same tissue region from the same time-point that these two genes were significantly upregulated in WT-infected mice when compared to KO-infected mice, thereby, complementing our findings from RNA-seq. Furthermore, at cecum and at small intestine sites, expression of IL-1β, IL-6, CCL2, and TNF-α were not different at either day in the two genotypes (II, Fig. S5). Therefore, qPCR data demonstrate increased inflammation in wild-type mice in large intestine is limited to earlier in *Salmonella* infection, while in other locations there is not genotype-dependent differences in expression of analyzed cytokines.

6 Discussion

Gastrointestinal inflammation, as in IBD and enteric infection, remains an intransigent clinical problem due to its complex and poorly defined molecular networks. One of the gaps in our current understanding remains the role of PARP14, an ADP-ribosyltransferase involved in cytokine signaling and in gut inflammation regulation. We demonstrate in this work evidence in healthy and inflamed IBD colonic tissues of marked PARP14 expression characterized by intense staining of epithelial cells. In DSS-induced colitis and *Salmonella* infection models in mice, PARP14 is induced upon inflammation. Deficiency in PARP14 is later accompanied by exaggerated tissue damage, impaired regeneration of epithelium, and impaired expression of inflammatory mediators at the level of transcripts. By elucidating these observations, we highlight PARP14 as a key molecular controller of gastrointestinal inflammation.

6.1 PARP14 Expression in Gastrointestinal Inflammation

Our study found out that PARP14 is expressed in the gastrointestinal tract of humans and mice, and that its expression is dynamically regulated during inflammatory conditions. Our study depended a lot on the PARP14 antibody (sc-377150, Santa Cruz Biotechnology) which we validated in-house for its specificity towards both human and mouse PARP14. In human colon biopsies, we observed that PARP14 protein localization was predominantly in the epithelial cells (both surface and crypt epithelium, most evidently in the surface), with some expression in lamina propria cells. This pattern was observed to be true across non-IBD controls and patients with ulcerative colitis (UC) or Crohn's disease (CD). While we noted a slight trend toward lower epithelial PARP14 staining in CD patients compared to controls, no statistical support was noted. We also noted a trend towards higher cryptal PARP14 staining in UC patients as compared to controls, but this was also not statistically significant. Importantly, within areas of active inflammation (e.g., UC biopsies with goblet cell loss and immune infiltrates), some areas of epithelial cells showed particularly intense PARP14 staining. This indicates that PARP14 expression may be induced as part of the mucosal response to injury or inflammation of the gut. While we were

observing this staining pattern of PARP14 in human gut biopsy IHC stainings, in 2021, Argmann et al. published an article titled - Molecular Characterization of Limited Ulcerative Colitis Reveals Novel Biology and Predictors of Disease Extension (Argmann et al., 2021). One of their key findings was that, when they performed RNA sequencing analysis of biopsy specimens from UC patients, network analysis revealed PARP14 as a key driver of disease extension. Furthermore, they found higher PARP14 protein levels in inflamed biopsy specimens of patients with limited UC inflammation that subsequently extended. Their immunofluorescence staining images showed granular PARP14 in the lamina propria and in the colonic crypt epithelial cells, which was not the focus of their study, but this reinforced our findings. In our study, we had also anaylsed published microarray data from bulk human colon tissue (control, n=6 and UC, n=24, Arijs et al., 2009; control, n=8, CD and UC, n=15, Galamb et al., 2008), and found highly significant upregulation of PARP14 mRNA in UC and CD IBD cohorts. This was in line with our observation, as pointed out above, regarding the trend towards higher cryptal PARP14 staining in UC patients as compared to the controls.

In line with this, our *in vitro* experiments demonstrated that inflammatory cytokines can upregulate PARP14. Human epithelial cells (HeLa) and human macrophage-like cells (THP-1) greatly increased PARP14 expression upon stimulation with IFN-α or IFN-γ. Previous studies indicate that PARP14 is widely expressed in tissues, with particularly relevant expression in the immune system (B lymphocytes, macrophages, dendritic cells and T cells). Its expression is dynamically regulated by cytokines: IL-4 (a Th2 cytokine) upregulates PARP14 in macrophages and B cells as part of the alternative activation program, and importantly, interferons (IFNs) – especially IFN-α/β (Type I IFNs) and IFN-γ (Type II IFN) – strongly induce PARP14 as an interferon-stimulated gene (ISG) (Caprara et al., 2018; Grunewald et al., 2019; Iwata et al., 2016). This induction by IFNs places PARP14 in the category of "interferon-stimulated PARPs", alongside PARP9 and PARP12, which are upregulated during antiviral responses (Kar et al., 2024; Morone & Grimaldi, 2024; Ribeiro et al., 2024). Thus, while baseline PARP14 levels can be moderate, they can spike during immune activation. This is significant because it means PARP14 likely plays a role in shaping the outcome of those immune responses. In macrophages, for example, IFN-γ or toll-like receptor (TLR) agonists (like bacterial LPS or viral double-stranded RNA) can increase PARP14 expression (Caprara et al., 2018; Iwata et al., 2016). In line with that, experiments have shown that knocking down or knocking out PARP14 can dampen certain interferon-stimulated gene responses. Caprara et al. (2018) demonstrated that PARP14 regulates a subset of Type I IFN-induced proteins by controlling their accumulation in the nucleus. We also found using QuPath IHC staining quantitation that in the large intestine of WT mice, *Salmonella* infection increased the nuclear PARP14 staining at day 1, but, at day 5,

less staining was detected, although the overall cellular PARP14 staining increased from the resting state to day 1 and further to day 5. We interpret this as a sign of an early infection requirement for nuclear PARP14. In Caprara (et al., 2018), loss of PARP14 led to reduced expression or nuclear localization of specific antiviral effectors, and cells became more susceptible to infection by intracellular bacteria. In fact, PARP14-deficient cells infected with *Salmonella* Typhimurium had elevated bacterial loads and reduced expression of IFN-driven genes compared to wild-type, indicating that PARP14 normally helps mount an effective antibacterial state. This finding and our finding, directly ties PARP14 to the innate immune response to bacteria, likely through enhancing interferon signaling pathways (such as those governed by IRF3/IRF7 and STAT1). Thus, rather than a simple "on/off" for inflammation, PARP14 appears to tune the immune response. PARP14 could amplify beneficial defense programs (anti-microbial, interferon-mediated) while limiting excessive inflammatory signaling that could cause tissue damage (by biasing responses toward IL-4/Th2 profiles). Such regulation hints that PARP14 might act downstream of pattern-recognition receptor signaling and IFN signaling to modulate gene expression and inflammatory outputs. Thus, during flares of IBD or acute infections when interferons and other cytokines abound, epithelial cells and immune cells are likely driven to express more PARP14 as part of the host response.

In murine models, we similarly found that PARP14 expression in the gut is low under basal conditions but it is strongly upregulated with inflammation. In healthy mouse colon tissue, PARP14 protein was only mildly detectable. However, upon DSS-induced colitis or oral *Salmonella* infection, PARP14 levels rose markedly, especially in intestinal epithelial cells. In DSS-treated mice, colon crypt epithelium became strongly PARP14-positive, showing a granular cytosolic staining reminiscent of the pattern in human colitis samples. This granular staining has been reported also under various conditions in different cell types (Carter-O'Connell et al., 2018; Challa et al., 2025; Raja et al., 2025). Its in Raja et al., 2025, it is reported that PARP14 localizes in p62 bodies upon interferon-induced stress. We also detected PARP14 in some F4/80^{+} colonic macrophages of DSS-treated mice, indicating that both epithelial and myeloid lineages contribute to PARP14's presence in the inflamed colon. Notably, the upregulation of PARP14 with DSS was evident in FVB/n mouse strain and less evident in C57BL/6N, suggesting a strain-dependent response. Consistent with protein data, Parp14 mRNA tended to increase in inflamed colon tissue as well (significantly so in C57BL/6N DSS colitis). In *Salmonella*-infected mice, we observed a similar induction: by 5 days post-infection, crypt epithelial cells of the colon (and to some extent the cecum) showed intense PARP14 staining relative to its uninfected control mice. Single-cell RNA-sequencing data further indicated that among intestinal epithelial subsets, enterocytes and tuft cells express the highest levels of Parp14 transcripts. Tuft cells are chemosensory

epithelial cells which are involved in type 2 immune activation (Feng et al., 2024; Momoh et al., 2025; Yi et al., 2019), so their increased Parp14 expression raises interesting questions about the role of PARP14 in epithelial-immune crosstalk. Both human and mouse data suggest PARP14 is a gene expressed as part of mucosal response to inflammatory stress, and particularly within the epithelium. This is a novel finding, as previous research on PARP14 has largely focused on immune cells (e.g., macrophages and lymphocytes) and its roles as a transcriptional regulator in those cells. Our results highlight that gut epithelial cells are a major compartment of PARP14 expression during inflammation, implying that PARP14 might influence epithelial physiology and barrier function in disease states.

The inducible expression of PARP14 suggests that it could be part of a protective inflammation-induced feedback mechanism. Epithelial cells when under attack by pathogens or inflammatory cytokines not only mount defense responses (like producing antimicrobial peptides and chemokines) but also upregulate regulatory molecules that can modulate these responses. It is notable that PARP14's subcellular localization in epithelium was largely cytosolic with punctate/granular distribution. While the exact significance of this localization is unclear, PARP14 is a multi-domain protein that can shuttle between nucleus and cytosol; its presence in cytosolic granules could relate to sites of mRNA regulation or signal complex assembly (PARP14 has been reported to influence mRNA stability and to localize to focal adhesion complexes in other contexts (Vyas et al., 2014)). The robust induction of PARP14 by interferons in epithelial cells aligns with known IFN-driven genes that help regulate inflammation. Many interferon-induced genes in epithelia serve to amplify anti-pathogen defenses or to temper inflammatory signaling to prevent host damage. In this regard, PARP14 may act as a brake on inflammatory signaling pathways in these cells (as it does in macrophages, discussed below). Our finding that PARP14 is upregulated by IFN-γ (a Th1 cytokine elevated in Crohn's disease and infections) and by IFN-α (a type I IFN often elevated during viral infections or barrier disruption) positions PARP14 as a potential mediator of crosstalk between classic immune pathways and epithelial homeostasis.

In summary, we established that PARP14 is an inflammation-inducible protein in the gut, especially in the epithelium. These results contribute to the field by identifying PARP14 as a part of the mucosal inflammatory transcriptome/proteome, suggesting that PARP14 could be one of the intracellular regulators that epithelial cells (and infiltrating macrophages) deploy to cope with the stresses of gastrointestinal diseases such as IBD or infection. Given that no current IBD therapies directly target epithelial responses (Liu et al., 2021), understanding epithelial-expressed regulators like PARP14 is valuable. The next sections will discuss how the presence or absence of PARP14 affects the course of colitis and infection, shedding light on its functional role.

6.2 PARP14 Deficiency Exacerbates Colitis and Impairs Mucosal Barrier Integrity

A central finding of this thesis is that the genetic ablation of PARP14 renders mice more susceptible to colonic injury and inflammation. In the DSS-induced colitis model, PARP14-deficient mice showed significantly worse clinical and histopathological outcomes when compared to the wild-type (WT) littermates. While, WT and knockout (KO) mice, both lost weights similarly upon DSS exposure (indicating that DSS was effective in both the genotypes), the PARP14 KO mice exhibited earlier and more severe rectal bleeding. Increased rectal bleeding in KO animals became apparent by day 4 of DSS treatment and reached statistical significance by day 8, unlike in WT mice where the bleeding was less pronounced. This pointed to a heightened vulnerability of the PARP14-null intestinal mucosa to this kind of significant haemorrhagic damage. By the end of the experiment, colon lengths (an inverse indicator of colitis severity) were shortened in both the groups due to DSS treatment induced injury, but were not markedly different between the genotypes, suggesting that while the overall inflammation was substantial in all DSS-treated mice, specific aspects of tissue damage were exacerbated by PARP14 loss.

Histological analysis provided clear evidence that PARP14-deficient colons suffered more extensive tissue damage during colitis. Blinded scoring of H&E-stained colon sections revealed that KO mice had significantly greater epithelial erosion, goblet cell loss, and inflammatory cell infiltration in the colon compared to WT mice given DSS. In both the distal and proximal colon, the absence of PARP14 amplified the pathological hallmarks of DSS colitis. For example, goblet cell depletion, a typical feature of ulcerative colitis in humans, was more pronounced in PARP14 KO mice. Goblet cells produce mucus in the gut epithelial layer and their loss has been reported to compromise the mucosal barrier and exacerbate inflammation (W. Chen et al., 2018). Depletion of goblet cells is also identified as a characteristic feature of UC and it enhances disease severity by reducing the protective mucus layer. Thus, the greater goblet cell loss in PARP14 KO mice suggests that such mice have a weaker barrier and reduced ability to maintain mucosal defenses during inflammatory stress. We also observed denser immune cell infiltration of the colonic lamina propria of KO mice, which suggests a more vigorous inflammatory response (most likely as a result of increased recruitment of neutrophils and monocytes). Interestingly, in areas of inflammation, KO colons often showed large erosions of the epithelium and submucosal edema, whereas WT colons retained more intact epithelium. The overall pathology score (a composite of edema, erosion, goblet loss, and infiltrates) was significantly higher in PARP14-deficient mice, conclusively demonstrating that PARP14 deficiency worsens colitis severity.

These results indicate that PARP14 possibly plays a protective role in maintaining colonic tissue integrity during inflammatory induced injury. In essence, PARP14-deficient mice are “sensitized” to colitis, they respond with more severe disease. It suggests that PARP14 is required for effective mucosal healing or for limiting tissue damage in the face of inflammatory insults. One possible explanation is that PARP14 might help preserve the epithelial barrier. Our data showed KO mice had increased epithelial erosion and decreased goblet cells, both of which point to barrier compromise. In IBD, a defective epithelial barrier allows luminal microbes and antigens to invade the tissue, fueling a vicious cycle of inflammation (Qiao et al., 2025; Quansah et al., 2023). The PARP14 KO mice's more bleeding and ulceration hints that their epithelial layer may be more fragile when inflamed. This aligns with our IHC data that PARP14 is induced in the epithelial cells during DSS treatment, possibly an adaptive response to protect or repair the epithelium. Without PARP14, the epithelium might be less able to withstand damage or to regenerate quickly. Indeed, in the discussion of transcriptional data we will see that PARP14 KO colons failed to upregulate many cell division and repair genes after DSS, supporting the notion that these mice have impaired mucosal healing.

One clue comes from immunological profiling of the mice. We examined whether baseline differences in microbiota or immune cell populations could predispose PARP14 KO mice to worse colitis. 16S rRNA sequencing of fecal microbiota revealed no significant dysbiosis in PARP14-deficient mice under steady-state conditions, alpha and beta diversity of the gut microbiome were similar to that of WT mice. Thus, the worsened colitis cannot be explained by an underlying microbiome imbalance (unlike some genetic colitis susceptibilities that arise from microbial shifts). We also assessed immune cell composition by flow cytometry. Prior to DSS treatment, PARP14 KO mice had normal populations of major leukocyte subsets in the colon, blood, and spleen. During DSS colitis, both WT and KO mice showed the expected innate immune responses (e.g., increased neutrophils and monocytes in the colon). The colon-infiltrating immune cells were not significantly different between genotypes, suggesting that the degree of inflammatory cell recruitment was similar. However, interesting differences emerged in systemic immune responses. PARP14 KO mice had altered monocyte and T cell distributions in blood and spleen after DSS, e.g., fewer circulating inflammatory monocytes, and an unusual increase in circulating CD4 T cells, compared to WT. These systemic changes imply that PARP14 might influence how the body mounts or terminates immune responses under stress. The colon, as the primary site of injury, did not show gross immune population differences in KO vs WT, meaning the exacerbated pathology in KO is not simply due to more immune cells invading the colon. Rather, it likely reflects qualitative differences in the inflammatory response and the epithelial damage/repair dynamics.

In essence, the PARP14 KO mice illustrate a failure to protect the mucosal barrier during colitis. The "so what?" of this finding for IBD is that PARP14 appears to be an intrinsic safeguard for the intestine, a factor that helps the tissue tolerate inflammatory insult. This adds a new piece to the puzzle of IBD pathogenesis. Most research has focused on immune system deregulation (excess cytokines, T cell imbalance, etc.) in driving IBD. Our data spotlight the role of epithelial-intrinsic regulators like PARP14 in preventing excessive damage. The implication is that patients with lower PARP14 activity (due to genetic or environmental factors) might experience more severe mucosal injury during flares. Supporting this notion, we recall that in the human colon biopsies, areas with severe inflammation still had high PARP14 in the epithelium, perhaps indicating the body's attempt to counteract the inflammation. If PARP14 is overwhelmed or insufficient, the result could be uncontrolled epithelial loss and ulceration.

From a therapeutic standpoint, these results would caution against inhibiting PARP14 in contexts of intestinal inflammation. Interestingly, PARP14 is being explored as a drug target in oncology and allergic diseases, where its activity in macrophages and Th2 cells is associated with pro-tumor or pro-allergic responses (Qin et al., 2019). PARP14 inhibitors are under development to reprogram tumor-associated macrophages to a more inflammatory (anti-tumor) state. In fact, a selective PARP14 inhibitor (RBN-3143) had advanced to clinical phase I trial in 2022 for atopic dermatitis (a chronic allergic skin inflammation), aiming to see if blocking PARP14 can reduce inflammation in this condition. The rationale is that atopic dermatitis involves activated Th2 cells and macrophages in the skin; by inhibiting PARP14's pro-Th2, pro-survival effects on these cells, inflammation might be mitigated. Macrophages in atopic dermatitis lesions help sustain chronic inflammation, and PARP14 inhibition could push them toward resolving the inflammation. This underscores the notion that PARP14 sits at a convergence point of immune pathways relevant to chronic inflammatory diseases. If successful, it could pave the way for PARP14 inhibitors in other disorders such as asthma, fibrosis, or even certain cancers where IL-4/IL-13 signaling and macrophages are contributors. As mentioned above, *in vitro*, *ex vivo* and *in vivo* studies have reported its beneficial effects in various cancers, i.e., to reprogram to an anti-tumor state (Leshem et al., 2025; Schenkel et al., 2021; Sturniolo et al., 2024; Wong et al., 2023). While that strategy might be beneficial in cancer, our findings suggest that systemic blockade of PARP14 could worsen conditions like colitis by removing a critical brake on inflammation-induced tissue damage. In other words, PARP14 seems to promote a wound-healing, protective phenotype in the gut, which we would not want to inhibit during IBD. This dichotomy exemplifies the context-dependent roles of immune regulators: PARP14's pro-repair (and anti-inflammatory) function is

desirable in colitis, even though its pro-Th2, anti-inflammatory bias might be detrimental in cancer immunity or certain infections.

In conclusion, we demonstrated that PARP14 is essential for limiting colitis severity. PARP14-deficient mice serve as a model of aggravated colitis with impaired barrier maintenance. This finding emphasizes the importance of factors that support epithelial integrity and modulate inflammation , an area of growing interest since mucosal healing is the gold-standard goal for IBD therapy. Our data contribute to a better understanding of the molecular players in mucosal healing: we identify PARP14 as a positive influence on healing, which operates by preserving goblet cells, limiting erosion, and possibly orchestrating the proper inflammatory milieu for resolution. The next section will see if this protective role of PARP14 extends to an infectious colitis scenario and how the absence of PARP14 alters the outcome of host–pathogen interactions in the gut.

6.3 PARP14 Modulates the Balance Between Pathogen Clearance and Immunopathology in *Salmonella* Infection

We found that PARP14 deficiency affects the host response to enteric infection by *Salmonella enterica* serovar Typhimurium. In a streptomycin-pretreatment mouse model of *Salmonella* colitis, PARP14-knockout mice exhibited exacerbated intestinal inflammation compared to the wild-type mice, similar to the heightened colitis sensitivity seen with DSS treatment. Despite suffering worse inflammation and tissue damage, PARP14 KO mice harbored significantly (only by a close margin) lower numbers of viable bacteria in the gut at the peak of infection. In other words, the absence of PARP14 led to modestly effective bacterial clearance in the intestine, at the cost of increased immunopathology. By day 5 post-infection (p.i.), when *Salmonella* colitis is well-established, PARP14-deficient mice showed more severe colonic histopathology. Similar to DSS colitis, infected KO mice had more extensive epithelial erosion, loss of goblet cells, and massive immune cell infiltration in the colon compared to the infected WT mice. The inflammatory lesions in KO mice often extended deeper and were more confluent, indicating that the infection-triggered colitis was more destructive without PARP14. The fewer *Salmonella* colony-forming units (CFUs) in the colon but with higher inflammatory tissue damage suggests that KO animals were slightly better at reducing the luminal/pathogen load in the large intestine by that time point. These results could imply that the heightened inflammation in KO mice created a more hostile environment for the bacterial proliferation, for instance possibly via increased neutrophil activity and antimicrobial factors, thereby limiting bacterial survival. This interpretation is supported by the notion that aggressive inflammation can control

Salmonella in the gut – for example, inflammatory mediators like IL-22 drive production of antimicrobial peptides (such as calprotectin and lipocalin-2) that starve bacteria of nutrients like iron, aiding clearance (Griffin & McSorley, 2011). It appears that in PARP14 KO mice, the immune response to *Salmonella* tilted towards the "pathogen limiting" side of the spectrum.

Interestingly, at an earlier stage (day 1 p.i.), we observed that PARP14 KO mice actually had higher *Salmonella* burdens in the liver compared to WT. This suggests that very early in infection, the absence of PARP14 might impair some initial containment of bacteria, allowing more to disseminate to the liver. However, by day 5, the trend reversed in the gut, as discussed. This temporal pattern hints that PARP14 may contribute to early innate defenses (perhaps within macrophages that control dissemination), whereas later on its absence causes a robust inflammatory response that helps in clearing the bacteria locally. The net outcome at day 5 was that KO mice had mostly similar bacterial loads as WT in the mesenteric lymph nodes, spleen, and other organs, aside from the noted decrease in gut and an increase in liver at day 1. The data do not conclusively support a role for PARP14 in enhancing bacterial clearance, however, PARP14 deficiency did not lead to an uncontrolled systemic infection, on the contrary, by the end of the experiment, the KO mice had effectively kept systemic infection in check and even slightly reduced the intestinal bacterial counts. The cost, however, was exacerbated histological changes in the intestine (and potentially more risk of morbidity if the damage were to continue). Indeed, the KO mice appeared sicker clinically, with more signs of pain and hunched posture correlating with their severe colitis, although, we did not observe mortality in this acute model, it could be speculated that a longer-term outcome might be worse for KO mice due to the tissue injury.

These findings carry significant implications for understanding mucosal immunity in enteric infections and IBD. They demonstrate that PARP14 is a modulator that can tune down the intensity of inflammation. In its presence (WT mice), the *Salmonella* infection causes moderate colitis while allowing persistence of some bacteria. In its absence (KO mice), the reins are off: the immune system responds with fulminant inflammation, which is slightly effective at clearing bacteria but inflicts greater damage on the host's own gut tissue. This is reminiscent of the phenotype seen in other models where regulatory pathways are disrupted. For example, mice lacking IL-10 (an anti-inflammatory cytokine) develop severe hyperinflammatory responses to gut microbes and are highly susceptible to colitis. In *Salmonella* infection, transient blockade of IL-10 or regulatory T cells leads to accelerated clearance of the bacteria but at the expense of inflammation (Griffin & McSorley, 2011). Similarly, our PARP14 KO mice can be thought of as lacking an internal "regulatory switch." PARP14, in this context, appears to normally act to restrain the proinflammatory response, thereby preventing immunopathology even

if that means the pathogen isn't cleared immediately. This balance is crucial in many infections: "the ability of *Salmonella* to persist depends on a balance between immune responses that clear the pathogen and avoidance of host damage" (Ruby et al., 2012). Our data empirically support this statement, WT (with PARP14) represent a balanced approach (some damage, some persistence), whereas KO (no PARP14) represent modest clearance with increased damage.

Delving into possible mechanisms, one likely role of PARP14 in infection is similar to what we posited in colitis: regulating immune cell activation and epithelial responses. PARP14 is known to influence macrophage polarization and cytokine production. *In vitro* studies have shown that PARP14 suppresses pro-inflammatory M1 gene expression and promotes M2 (alternative, tissue-reparative) gene expression in macrophages (Fehr et al., 2020). Specifically, PARP14 inhibits the IFN-γ/STAT1 signaling axis while enhancing IL-4/STAT6 responses. In the context of *Salmonella*, which elicits strong Th1/IFN-γ responses, PARP14 would be expected to modulate STAT1-driven inflammatory programs. Consistent with this, we observed that PARP14-deficient mice exhibited significantly lower levels of IL-6, IL-1β, and TNF-α on day 1 post-infection, suggesting a dampened early innate response. However, by day 5, colonic cytokine levels were comparable between genotypes, and interestingly, bacterial burden in the colon was slightly lower in KO mice. This suggests that reduced early cytokine production did not impair and may even have favored bacterial clearance in the colon. Interestingly, despite the dampened early response, KO mice exhibited significantly higher immune cell infiltration histoscore in the colon on day 5. This suggests a delayed but ultimately exaggerated immune recruitment, potentially reflecting a loss of regulatory control in the absence of PARP14. Such dysregulation could lead to enhanced tissue infiltration even after minimal bacterial clearance is achieved. These findings point to a nuanced role for PARP14, not in initiating inflammation per se, but in modulating the timing and magnitude of immune responses during mucosal infection.

It is instructive to consider the specific observation of goblet cell loss in infected KO mice. By day 5, PARP14 KO mice had a notable absence of goblet cells in the colon compared to WT. Goblet cells and the mucus layer are critical for tolerating luminal bacteria; without mucus, bacteria make more direct contact with the epithelium, intensifying the immune response (Qiao et al., 2025). The severe depletion of goblet cells in KO might be both a cause and consequence of the intense inflammation. Inflammatory cytokines like IL-18 can impair goblet cell function and mucus production (Nowarski et al., 2015). Furthermore, an inability to regenerate goblet cells (due to impaired differentiation signals) would mean the mucus barrier cannot be restored. There is evidence that type 2 cytokines (IL-4, IL-13) promote goblet cell differentiation and mucus production as part of healing. If PARP14

fosters IL-4/IL-13 signaling (via STAT6 co-activation), then its absence could leave a deficit in those pro-goblet, tissue-restorative signals. In line with this, our DSS model data hinted that PARP14 KO mice might struggle to induce reparative pathways. In infection, the lack of PARP14 could similarly skew the immune response away from any balanced response that includes IL-4/IL-13 activity, thus failing to protect goblet cells.

Another aspect is the architectural genes that PARP14 KO failed to upregulate, which we discovered in *Salmonella* model. Bulk RNA-seq analysis of infected colonic tissue suggested that PARP14-deficient mice lacked certain gene expression signatures related to cell adhesion, cytoskeletal remodeling, and tissue architecture that were present in WT during infection. For instance, pathways like focal adhesion and tight junction assembly were downregulated in infected KO tissue. Many of the affected genes (e.g., fibronectin- Fn1, radixin- Rdx, myosin light chain- Myl9, etc.) are involved in maintaining the structural integrity of the epithelial barrier and enabling cells to migrate and close wounds. The implication is that without PARP14, the infected epithelium might not properly activate the programs needed to preserve or repair the mucosal barrier. This mechanistic insight aligns perfectly with the histological outcome: KO mice showed extensive epithelial ulceration, consistent with but not conclusive for barrier dysfunction in the face of infection. In contrast, WT mice, aided by PARP14, activated those cell adhesion/cytoskeletal pathways which likely helped limit the damage (for example, by strengthening cell junctions or promoting epithelial cell migration to cover denuded areas). This highlights a dual role for PARP14 during infection on one hand restraining excessive inflammation, and on the other hand facilitating tissue-protective responses. Together, these roles allow PARP14-sufficient hosts to strike a better balance: moderate inflammation that is sufficient to control the pathogen (though not completely eradicate it by day 5), while sustaining the epithelial barrier to avoid catastrophic tissue loss.

From a research perspective on IBD, these infection outcomes are enlightening. Most models of pathogenesis in IBD involve a dysregulated response to intestinal bacteria, where insufficient immune response makes one susceptible to the commensal microbiota or excessive response leads to chronic damage. We model the latter, an over-reactive tendency to a challenge by bacteria, in PARP14 KO. Interestingly, although *S.* Typhimurium is an acute pathogen and a non-commensal, how the KO immune system responds may tell us something of how the immune system of an IBD patient responds to enteric microbes. If PARP14 performs similar functions in humans, one would then predict that individuals of decreased PARP14 activity would be susceptible to increased inflammatory responses to enteric infection or microbiota and IBD flare-ups. It also suggests that treatments which increase immune clearance (e.g., cure infection) need to be counterbalanced by ones that prevent tissue damage, exactly what PARP14 naturally does.

In addition, the *Salmonella* model emphasizes the requirement for protective mechanisms for immunopathology. Therapies for IBD generally dampen the immune system overall (e.g., anti-TNF, steroids) to prevent tissue destruction. Our results indicate the possibility of more selective strategies: activating or recapitulating PARP14 function may ensure a well-tuned immune response that destroys the pathogen without excessive collateral damage. Of course, further experiments are needed to address whether augmentation of PARP14 function would be beneficial during infection or flare (the inverse of our KO experiment). However, these data identify PARP14 as a critical checkpoint in mucosal immunity that keeps the host-pathogen struggle in check.

In conclusion, we've demonstrated that PARP14 deficiency leads to a dysregulated host response to infection characterized by hyper-inflammation and slightly enhanced bacterial clearance. The KO phenotype definitively shows the principle of immunopathology, where both the defense response and the pathogen are guilty of causing disease. PARP14 is revealed to be an element that operates to exclude this possibility by favoring a response that is adequate to clear the pathogen in the end, yet insufficient to ravage the host tissue in the process. These discoveries are contributory to the comprehension of GI inflammation as it uncovers PARP14 as a potential defense against "friendly fire" in immune responses to enteric microbes. It confirms that treatments for inflammation in the intestines will be compelled to balance the control of the pathogen and the preservation of tissue, the very balance that PARP14 is revealed to favor.

6.4 PARP14-Dependent Transcriptional Signatures in Mucosal Inflammation

In trying to better comprehend how PARP14-deficiency caused severe disease outcome, we compared gene expression signatures (transcriptional signatures) in the tissues of our mouse models. We were able to identify the molecular pathways governed by PARP14 upon inflammatory stimulus in the gut. We made two relevant comparisons: (1) DSS-treated PARP14 KO versus WT colon, which approximates how PARP14 modulates the colonic response to insult, and (2) *Salmonella*-infected KO versus WT intestine, which approximates PARP14 effects on host response to infection. Regardless of infection and chemical colitis variations, we observed consistent patterns across both conditions. In both cases, PARP14 deficiency led to the failure to induce specific protection gene programs (involving cell proliferation, adhesion, and cytoskeletal organization) and led to over-representations of inflammatory response genes. These signatures correspond to the associated physiological phenotypes, providing a molecular rationale for the heightened susceptibility of PARP14 KO mice.

In the DSS colitis model, bulk RNA sequencing of distal colon tissue at the peak of disease (day 8) revealed that PARP14 has a profound impact on the transcriptional landscape of the inflamed colon. Notably, Parp14 itself was upregulated ~3-fold in WT colons after DSS, confirming that PARP14 is part of the typical transcriptional response to DSS-induced colitis (and supporting our earlier protein data). When we examined differentially expressed genes (DEGs) between WT and PARP14 KO (both DSS-treated), it was evident that the WT colons activated many gene sets that the KO colons did not. Gene ontology (GO) enrichment analysis suggested that WT mice responded to DSS by upregulating a plethora of genes associated with DNA replication, cell division, and cell cycle. This makes sense biologically: after epithelial injury, healthy tissues attempt to regenerate. The WT mice were mounting a proliferative response, presumably to replenish the epithelial layer.

In stark contrast, PARP14 KO mice under DSS showed an upregulated gene profile dominated by inflammatory and immune response pathways. The top GO terms in DSS-treated KO colon were all related to inflammation (e.g., innate immune response, cytokine production, chemotaxis). This indicates an exaggerated or dysregulated inflammatory transcriptional response in the absence of PARP14. At the same time, the KO mice failed to induce the robust proliferative and cell cycle programs that WT did. The KO's transcriptome lacked many of the cell division-related GO terms seen in WT, and overall had far fewer genes in the "tissue repair/regeneration" category. In fact, when looking at downregulated DEGs in KO (genes that were expressed less in KO than WT after DSS), there were relatively few enriched GO/KEGG categories, implying that KO mice simply did not turn on many pathways that WT did. Taken together, these data paint a picture in which PARP14-deficient colons mount a primarily inflammatory response to injury, with insufficient activation of compensatory proliferative/healing responses. This skewed transcriptional program likely underlies the greater tissue damage observed: without strong cell renewal, the epithelium cannot repair lesions, and unchecked inflammation further worsens the damage.

Strikingly, we also found that even in the resting state (without DSS), PARP14-deficient colons showed an altered transcriptome. Prior to any treatment, KO vs WT comparison revealed that KO mice had significantly lower expression of a set of genes related to inflammation/immune responses. Specifically, 78 genes were downregulated in KO at baseline, and GO analysis of these pointed to functions in innate immunity and host defense. About 44% of these downregulated genes fell into 12 GO categories all tied to immune or infection responses. Additionally, hundreds of genes were expressed in WT colons but essentially absent in KO colons; a KEGG analysis of those missing genes showed many belonged to inflammatory or pathogen-response pathways. This was a striking finding, PARP14 deficiency "silenced" a subset of immune genes in the colon under basal conditions. One

interpretation is that PARP14 may help maintain a certain level of immune preparedness in the gut mucosa. Without it, expression of some chemokines, receptors, or antimicrobial peptides might be lower, possibly making the tissue initially less responsive to stimuli. This could explain why at day 1 of *Salmonella* infection, KO mice had higher bacterial dissemination (they might have started with a muted immediate response due to these baseline deficits). However, as the infection or DSS injury progressed, other compensatory signals (possibly from massively activated immune cells) kicked in, leading to the eventual hyperinflammation in KO mice. Another possibility is that those baseline differences reflect PARP14's role in shaping the microbiome or tonic immune stimulation; however, as noted, the microbiota composition appeared normal, so it might be more about intrinsic signaling pathways.

In the *Salmonella* infection model, although we did not perform an extensive separate RNA-seq in this thesis beyond identifying key signatures, the data we have are consistent with the DSS findings. PARP14 KO intestines appeared to lack proper induction of structural and barrier-related genes during infection. The downregulation of focal adhesion, tight junction, and actin cytoskeleton pathways in infected KO mice suggests that PARP14 normally contributes to upregulating these pathways when the gut is infected. This suggests a common core function of PARP14 in any GI inflammatory context: promoting genes that maintain tissue architecture and barrier function. The functional consequence is clear – without those gene programs, KO mice cannot effectively preserve their epithelial integrity, leading to the erosions and ulceration observed.

These molecular findings substantially advance our understanding of PARP14's function. Prior to this work, there was evidence of PARP14 regulating the immune cell functions *in vitro*, but its role in the gut was undefined. We now have evidence that PARP14 is a regulator of gene expression in the context of intestinal inflammation. It affects a broad network of genes governing inflammation intensity, cell survival, and epithelial restitution. This positions PARP14 as a kind of "molecular switch" that can toggle the inflammatory response between a destructive mode and a healing mode.

From a "big picture" standpoint, these transcriptional signatures underscore a fundamental point for IBD: successful resolution of inflammation requires a timely shift from inflammatory gene expression to regenerative gene expression. In our WT mice, that shift was occurring (they induced cell cycle genes alongside immune genes). In PARP14 KO mice, the shift was incomplete or absent since they remained stuck in the inflammatory gear. Therefore, PARP14 might be one factor that facilitates the switch to a healing program, perhaps by engaging transcription factors like STAT6, PPARγ, or others that drive mucosal healing. Its ADP-ribosyltransferase activity could modulate key regulators (for instance, PARP14

ADP-ribosylates STAT1 which can reduce STAT1's ability to drive gene expression). It may also act as a co-activator/repressor without its enzymatic function, as suggested in studies showing that PARP14 can bind promoter regions and influence transcription complexes.

There is another interesting approach in speculating the role of PARP14, that is its involvement in mRNA stability. One study reported that *Parp14* knockout macrophages exhibit specific alterations such as increased Tissue factor (Tf) expression upon stimulation. It also found that PARP14 reduces the stability of Tf mRNA in LPS-activated macrophages. Tissue factor is a pro-coagulant protein often induced in M1-polarized macrophages that contributes to thrombosis and plaque instability in atherosclerosis. The observation that PARP14-deficient macrophages produce more Tf suggests that PARP14 normally restrains certain pro-inflammatory, pro-thrombotic activation programs (Iqbal et al., 2014). In line with an anti-inflammatory role, PARP14 might be protecting against chronic inflammatory damage by keeping macrophages in check. PARP14 could similarly target certain mRNAs in epithelial or immune cells in the colon, perhaps destabilizing transcripts of inflammatory mediators. If PARP14 normally helps degrade proinflammatory mRNAs, its absence could lead to a prolonged expression of those cytokines which is consistent with our observation of sustained inflammatory gene expression in KO. This could be another mechanism behind the transcriptional differences that we observed.

In summary, our investigation into the molecular signatures of the colon revealed that PARP14 deficiency influences skewing of the gene expression balance towards inflammation and away from tissue maintenance. On the contrary, PARP14 presence correlates with the appropriate induction of barrier-protective and wound-healing genes during colitis or infection. These data not only explain the phenotype of the mice at a mechanistic level but also highlight specific pathways that PARP14 controls. For instance, the focal adhesion and tight junction pathways that were impaired in KO might be targeted in future to see if boosting those can compensate for lack of PARP14. It also provides a list of potential downstream effectors of PARP14, e.g., components of epithelial proliferation or epithelial defense might be among those influenced by PARP14 and worth exploring.

As a whole, evaluation of our transcriptomic findings supports the notion that PARP14 possibly operates as a molecular mediator linking pro-inflammatory signals (including IFNs and cytokines) to genomics that dictate the outcome of inflammatory responses. By activating cell-repair programs and modulating inflammatory programs, PARP14 redirects the tissue away from destruction and towards repairing. This holistic perspective, and that too from *in vivo* models, is perhaps the most significant contribution of this thesis, as it positions PARP14 as a potential key gene expression-level regulator in GI IBD pathology.

6.5 Limitations and Future Directions

Even with our contribution towards novel key information in our research on PARP14's role in gastrointestinal inflammation, we must also address its shortcomings. Our human data for IBD were derived from a sample size of 9 controls, 12 UC, 7 CD, making a cohort of 28 patients. Such sample size was perhaps too low to detect differences in PARP14 expression in UC vs. CD, for example, in correlations with disease severity. A larger patient sample size would determine whether PARP14 expression is dysregulated in some subset of IBD patients, and within specific histologic features. In future, we would be keen to investigate PARP14 genetic variants in IBD patients. Genome-wide examinations would determine whether any polymorphisms in PARP14 were implicated in susceptibility to, or in severity of, IBD. Such information would address directly our data's relevance to human disease, e.g., loss-of-function variant in PARP14 predisposing to severe colitis. Further, characterization of PARP14 expression in intestinal organoid culture, or in *ex vivo* biopsy stimulated by specific agonists, would translate *in vivo* mouse observations into human tissue responses.

Regarding the mouse models, one limitation is the use of global PARP14 knockout mice. While this allowed us to see the overall effect of PARP14 loss, it does not pinpoint which cell types are responsible for the observed phenotypes. PARP14 is expressed in many cells (epithelium, macrophages, possibly T cells), and any or all of these could contribute to the colitis and infection outcomes. For instance, is the exacerbated DSS colitis mainly due to lack of PARP14 in epithelial cells (leading to impaired barrier repair), or due to lack in macrophages (leading to excessive inflammatory signaling), or both? Our data suggest both compartments play a role, but to dissect this, cell type-specific knockouts will be needed. Future studies could generate mice with PARP14 deleted only in intestinal epithelial cells (using Villin-Cre) or only in myeloid cells (LysM-Cre) or other immune cells. Comparing such conditional KOs would clarify where PARP14 is essential. For example, an epithelial-specific PARP14 KO might recapitulate the barrier integrity issues (goblet cell loss, erosions), whereas a myeloid-specific KO might recapitulate the excessive cytokine production and immune cell infiltration. It is also possible that PARP14 acts in non-hematopoietic vs hematopoietic compartments in a complementary fashion. Bone marrow chimera experiments (transferring KO or WT marrow into opposite genotype hosts) could further parse this out by showing if the primary driver is bone marrow-derived cells or tissue resident cells.

Another limitation is that our functional experiments were only done in DSS and acute *Salmonella* models. While this is appealing, colitis in DSS causes largely acute epithelial damage and activation of innate immunity (Laroui et al., 2012); it may not reflect the entirety of chronocity or adaptative immune components of IBD. And our

Salmonella infection is acute and primarily innate-mediated (Barthel et al., 2003). Future attempts at elucidating PARP14's function in chronocity or an adaptative immune-mediated colitis would be of interest. For instance, IL-10 deficient colitis or colitis in T cell transfer (where CD4 T cells cause chronocity in immunodeficient animals) might clarify PARP14's function in regulation of Th2-biased inflammation driven by T cells. Since PARP14's known function is to induce Th2 at expense of Th1 responses in differentiation of T cells (Mehrotra et al., 2013; Riley et al., 2013), one would predict in disease driven by T cells one would see loss of PARP14 resulting in Th1/Th17 bias and worsened colitis. Preliminary literature data suggests PARP14 can regulate production of cytokines in T cells (e.g., enhancing IL-4 in Th2 cells). Trying this in an IBD context is an intuitive next step. Investigating other infection models further would be worthwhile. Intestinal pathogen *Citrobacter rodentium* in mice causing colonic mucosal pathology and requiring Th17 responses for clearing might be an interesting one to test whether loss of PARP14 causes similar trade-offs in clearance vs pathology (Omenetti et al., 2019; Z. Wang et al., 2014). Another avenue is helminth infection (e.g., *Trichuris muris* in colon), causing Th2 responses and goblet cell hyperplasia (Klementowicz et al., 2012). PARP14 can be necessary for an efficacious Th2 response to eliminate worms (Cho et al., 2009; Goenka & Boothby, 2006); interestingly, PARP14 KO would be unable to clear an infection because it's unable to elicit an effective Th2 response. These experiments would also inform us about PARP14 functions in different types of mucosal immune responses (Th1/Th17 versus Th2-predominant).

In terms of mechanistic biochemical studies, our work did not distinguish whether the enzymatic activity of PARP14 is required for its protective role, or if it mainly acts as a scaffolding/co-regulatory protein. PARP14 has ADP-ribosyltransferase activity that could modify target proteins like STAT1. We inferred from literature that PARP14 likely ADP-ribosylates STAT1 to suppress its activity (Iwata et al., 2016). It would be a compelling future experiment to create a catalytically inactive PARP14 mutant mouse (knock-in) to see if it can still rescue the KO phenotype. If a catalysis-dead PARP14 fails to protect against colitis, that means the enzymatic activity is critical (perhaps ADP-ribosylation of STAT1 or other factors is needed to dampen inflammation). If a catalysis-dead version still provides protection, then PARP14's role is more as a transcriptional co-regulator (for instance, as a component of STAT6 transcriptional complexes). Pharmacological tools could also be applied: a specific PARP14 inhibitor (some have been reported recently, as mentioned in section 6.2) could be used in WT mice to mimic the KO and see if it acutely worsens colitis, which would corroborate our genetic findings and also test drug effects. Conversely, finding a way to boost PARP14 activity or expression *in vivo* could have therapeutic potential. Perhaps agents that activate STAT6 (like IL-4/IL-13) indirectly engage PARP14's beneficial

effects, something that could be explored by treating KO and WT mice with an IL-4 analog or IL-33 (which promotes type 2 immunity) during colitis.

Another limitation to note is that our current analysis focused on gene expression and histological outcomes, but we did not measure the protein levels of many cytokines or effectors in the colon (aside from PARP14 itself). Future work should measure key inflammatory cytokines (TNF, IL-6, IL-1β, IFN-γ) and regulatory cytokines (IL-10, IL-22, IL-33) in KO vs WT colitis tissues. We expect the proinflammatory proteins to be higher in KO. Quantifying such differences would further explain how PARP14 shapes the cytokine milieu. Similarly, an assessment of epithelial proliferation (via Ki-67 staining) in DSS colons could explain the RNA-seq showing cell-cycle dependent pathways to be affected in KO.

Regarding RNA-seq analysis of *Salmonella* model, while it provided key insights into differential gene expression, it is important to acknowledge several limitations. Sequencing depth and sample size, particularly in the *Salmonella* model, may limit detection of subtle or low-abundance transcripts. Furthermore, pathway enrichment analyses rely on gene list cutoffs and database annotations that may not fully capture context-specific regulation. The possibility of false positives in differential expression or pathway assignment cannot be ruled out. Therefore, pathway-level interpretations should be considered indicative rather than definitive.

From a translational perspective, our findings open up the question: could enhancing PARP14 activity be a therapeutic strategy in IBD? While direct activation of an ADP-ribosyltransferase is not straightforward, perhaps stabilizing PARP14 or preventing its inhibition could be beneficial. We saw that PARP9 may act as a negative regulator of PARP14 in macrophages (Iwata et al., 2016). Targeting the PARP9-DTX3L complex (which in some contexts opposes PARP14) might tilt the balance in favor of PARP14's anti-inflammatory action. However, this is speculative and would require careful assessment given the diverse roles of these molecules in different diseases.

Finally, an applied constraint was that, as our work was the first to investigate PARP14 in gut inflammation in 2017, and a lot of ground had to be made. As such, some mechanistic questions remain outstanding (as discussed above). Future research will likely include further targeted mechanistic experiments (e.g., identifying direct PARP14 substrates within colon tissue in colitis, perhaps using mass spec-based ADP-ribosylation proteomics). It would be of interest to probe PARP14's interacting partners within intestinal cells. Mass spec and co-IPs from colon tissues can determine in which PARP14-containing complexes PARP14 is engaged during inflammation (does it partner with NF-κB, STATs, chromatin remodels, etc., within epithelial vs immune cells?). In addition to that, bulk-tissue RNA-sequencing and leukocyte subclass population analysis by multicolour flow cytometry, were very critical in helping us understand the immune environment of

the colon, but did not help us to understand the role of each cell type. Bulk transcriptome analysis of intestinal tissues is hampered by its inability to separate out contributions of individual cell types: expression levels are averaged over a heterogeneous mixture of epithelial, stromal, and immune cells, masking cell-type-specific signals, diluting rare but causally implicated populations (e.g., activated lamina propria T cell subpopulations), and confounding cell compositional variations with true transcription changes. Leukocyte population analysis by flow cytometry reveals just the number of a particular subset of cells in that single-cell population from a bulk tissue, and it does not help in understanding the function of those subsets. For e.g., in our DSS-treated mice, colon tissue showed no significant change in leukocyte subclass population analysis between WT and KO DSS-treated mice, in spite of severe tissue damage as shown by histopathology. Single-cell transcriptomics resolves this cell heterogeneity by sequencing thousands of individual cells to identify disease-associated cell states (e.g., pro inflammatory macrophage subtypes or stressed epithelial stem cells), reconstructing differentiation pathways across the inflamed mucosa, and mapping intercellular conversation networks. By revealing the true cellular origins of inflammatory mediators as well as responses to treatment or infection by individual cell populations, single-cell methods have the potential for more accurate biomarkers and personalized therapies for intestinal diseases (Gudiño et al., 2025). In conclusion, it would be imperative to understand the reason behind severe disease states in the absence of PARP14. Is the severity driven by malfunctioning epithelial cells, that cause breach of intestinal epithelial barrier causing inflammatory cascades, or, is the microbiota disturbed in the disease states, causing epithelial damage and inflammatory cascades, or, are the immune cells malfunctioning, resulting in excessive tissue damage (Fig. 9)?

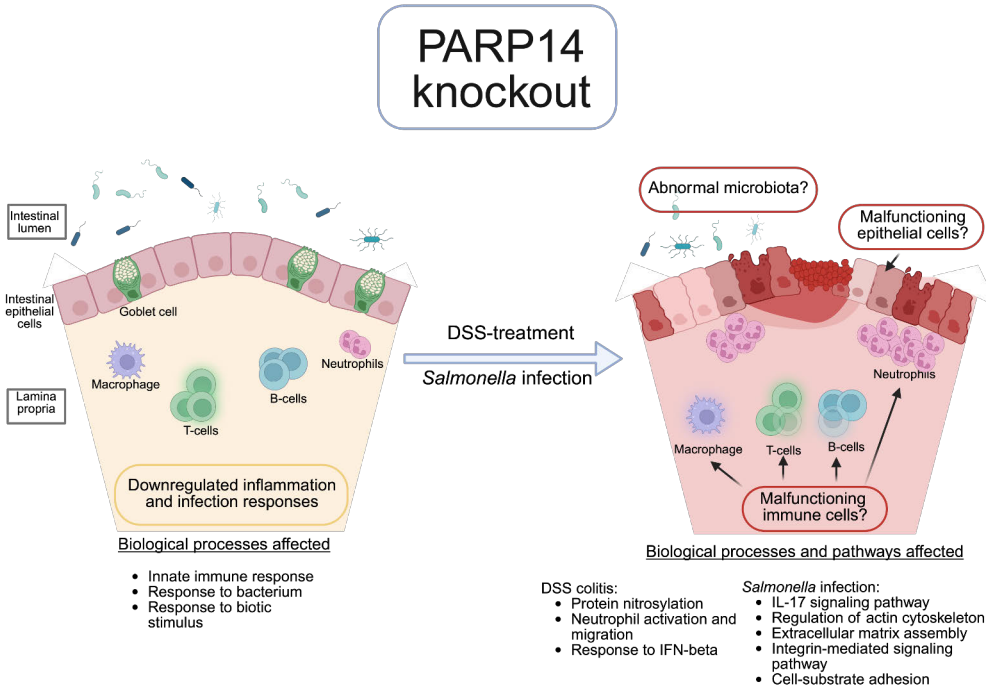


Figure 9. Simple illustration of PARP14-deficient colon. In the resting state (left), PARP14 knockout (KO) mice showed normal tissue architecture. RNA-seq had revealed downregulated inflammation and infection response biological processes and pathways. Under stress (right) of both DSS-colitis and *Salmonella* infection, severe tissue damage was observed when compared to inflamed WT tissue- increased epithelial erosion, goblet cell loss and immune cell infiltration. RNA-seq revealed inflammatory responses, cell adhesion, and cytoskeleton pathways and processes to be affected. Illustration created with biorender.com.

7 Conclusions

Of the 17 highly conserved members of the PARP enzyme family that executes mono- and poly- ADP-ribosylation of target substrates, different studies of PARP1, PARP2, and PARP5a/5b have been launched in the past decades. Growing curiosity in the field regarding remaining PARPs, led to further investigations and PARP14 was found to be regulating multiple cellular responses and immune signaling pathways. Its role in gastrointestinal inflammation - especially during IBD and *Salmonella* infection- is, however, unclear. With an extensive utilization of human colonic tissue and robust murine models, such as those involving DSS-colitis and *Salmonella* infection, this thesis study shows PARP14's multifaceted role in regulating inflammation, promoting mucosal integrity, and orchestrating well-balanced immune responses.

The initial experiments in human colon biopsies showed PARP14 to be compartmentalized largely in intestinal epithelium compartments. Immunohistochemical staining revealed surface and crypt epithelial cells to be positive for PARP14 in non-IBD, ulcerative colitis and Crohn's disease patient biopsies, with no changes in staining intensity between non-IBD and IBD groups. This observation was followed by *in vitro* experiments in human epithelial (HeLa229) and macrophage (THP-1) cell lines. In these cells, pro-inflammatory cytokines IFN-α and IFN-γ caused robust induction of PARP14 in a time-dependent manner, further strengthening the established role of PARP14 as an interferon-stimulated gene (ISG).

Basal PARP14 in healthy mice was low, but following inflammation challenge by DSS or *Salmonella*, its expression was increased, particularly in intestinal epithelium as observed by immunohistochemical staining. Careful image inspection of FFPE sections confirmed not just an increase in intensity of PARP14 following inflammatory challenge, but also in its subcellular localization, suggestive of active participation in stress and repair signaling. Quantitative PCR and single-cell RNA-sequencing analyses confirmed this by showing increased PARP14 transcripts. Single-cell RNA-seq analyses specifically showed increased PARP14 in the epithelial subtypes such as enterocytes and tuft cells following infection.

One of the key conclusions of this research is the functional significance of PARP14 loss in gastrointestinal injury. From its use in both the DSS colitis and the streptomycin-pretreated *Salmonella* infection model, we found that absence of PARP14 resulted in worse mucosal injury. With use of the DSS model, PARP14-deficient mice showed extensive rectal bleeding, increased epithelial erosion, widespread goblet cell loss, and a heightened infiltration of inflammatory cells relative to wild-type mice. These observations are compelling evidence for PARP14 playing a protective role by inhibiting tissue damage and maintaining barrier function in the state of inflammatory stress.

Similarly, in *Salmonella* infection, PARP14-deficient mice had an increased state of inflammation. Even though an increased level of inflammation was correlated with mildly increased clearance of bacteria from the gut, the concomitant tissue destruction, evidenced by extensive destruction of the epithelial structure and decreased goblet cells, underscored an intrinsic balance necessary for pathogen clearance versus mucosal integrity. Overall, these data point to PARP14 as an indispensable immunoregulatory checkpoint, inhibiting unnecessary pro-inflammatory signaling while promoting mechanisms of restitution in the epithelium.

At the level of transcription, our RNA sequencing experiments have demonstrated novel signatures further attesting to PARP14's protective role. In wild-type mice subjected to DSS-induced colitis, gene expression profiles were enriched for cell cycle advancement and tissue regeneration pathways, consistent with an active process of repair. In PARP14-deficient mice, in contrast, the transcriptome was dominated by genes involved in inflammation while having significantly poorer representation of reparative gene programs. Such an imbalance in gene expression attests to PARP14's critical role in enforcing a switch from an inflammation to a regeneration mode in the gut upon inflammation.

In summary, these observations elucidate PARP14 as a central regulatory molecule to integrate cytokine signaling, modulate innate and adaptive immunity, and preserve mucosal homeostasis in gut inflammation. PARP14 keeps the gut healthy by integrating tissue-protective and cell-cycle gene programs and suppressing pro-inflammatory mediators to dampen inflammation without triggering uncontrolled tissue damage. Lastly, despite its limitations, this thesis clearly demonstrates the novel function for PARP14 in the protection of gut from inflammatory injury. It gives rise to many follow-up studies to fully untangle the cell-type-specific activities and molecular processes of PARP14. Further investigations will not only fill the gaps of our research but also potentially pave the way for new therapeutic directions. If we can confirm that augmentation of PARP14's pathway promotes mucosal healing and suppresses harmful inflammation, we may reveal new methods of treating IBD, methods that complement existing

immunosuppressive therapies by stabilizing the mucosal barrier and hastening resolution of inflammation. This line of research, thus, based on this thesis, holds promise for both immunologic discovery and translation to the clinic for gastrointestinal inflammation.

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