Enterobacterial colonization of enteroids and colonoids

Interplay with the epithelial surface and inflammasomes

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Abstract

Geiser, P. 2024. Enterobacterial colonization of enteroids and colonoids. *Interplay with the epithelial surface and inflammasomes*. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 2081. 87 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-2248-3.

The model enteropathogens *Salmonella enterica* serovar Typhimurium (*S*.Tm) and *Shigella flexneri* (*S. flexneri*) colonize the intestinal lumen and target the apical surface of intestinal epithelial cells (IECs) for invasion. Intraepithelial bacterial populations are restricted by IEC inflammasomes, cytosolic multiprotein complexes that assemble upon detection of bacterium-derived molecules and induce infected IEC death and extrusion. The recent development of non-transformed, purely epithelial enteroid and colonoid models has opened up venues for physiologically relevant mechanistic studies at high spatial and temporal resolution. In this thesis, we employed live-cell imaging in enteroids and colonoids to study how virulence factor modules governing host cell surface targeting and interaction with inflammasomes shape *S*.Tm and *S. flexneri* epithelial colonization strategies.

In **Paper I**, we found that *S*.Tm invasion induced tissue-scale epithelial contractions via activation of the IEC-intrinsic NAIP/NLRC4 inflammasome, and myosin-based propagation of the contractile response to surrounding IECs. This response preceded infected IEC extrusion and resulted in local tissue compaction to prevent loss of epithelial integrity during infection. In **Paper II**, enteroid microinjection was employed to map the stages of *S*.Tm epithelial colonization and identify luminal expansion and cycles of IEC invasion, intraepithelial replication and infected IEC extrusion as collaborative pathways to ensure efficient colonization of both luminal and epithelial niches. In **Paper III**, parallel enteroid and colonoid infections characterized an *S*.Tm apical targeting module comprised of flagellar motility and the SPI-4-encoded SiiE adhesin system to foster abundant, but short-lived, invasion foci, terminated by prompt induction of IEC death and extrusion. Low *S. flexneri* invasion efficiency, in contrast, is compensated by an intraepithelial expansion module coupling OspC3-mediated inflammasome suppression with IcsA-driven lateral spread to evade restriction by IEC death and extrusion. In **Paper IV**, we developed a simplistic high-throughput infection model to assess host cell surface determinants involved in *S*.Tm targeting. Chemical cell surface manipulations and ectopic glycoprotein expression established that glycocalyx constituents and bacterial cell surface appendages represent size-dependent steric barriers towards invasion. Hence, while adhesins are necessary to penetrate the apical IEC glycocalyx, a naked host cell membrane rich in cholesterol would be the preferred surface for *S*.Tm targeting.

Keywords: bacterial infection, Salmonella, Shigella, intestinal epithelium, enteroids, colonoids, inflammasomes, mucins, glycocalyx, live-cell imaging

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To my children Joan and Joëlle,
never stop exploring the world around you.

List of Papers

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

I.   Samperio Ventayol, P., **Geiser, P.**, Di Martino, M. L., Florbrant, A., Fattinger, S. A., Walder, N., Sima, E., Shao, F., Gekara, N. O., Sundbom, M., Hardt, W.-D., Webb, D.-L., Hellström, P. M., Eriksson, J., Sellin, M. E. (2021) Bacterial detection by NAIP/NLRC4 elicits prompt contractions of intestinal epithelial cell layers. *Proc Natl Acad Sci U S A*, 118(16):e2013963118.

II. **Geiser, P.**, Di Martino M. L., Samperio Ventayol, P., Eriksson, J., Sima, E., Al-Saffar, A. K., Ahl, D., Phillipson, M., Webb, D.-L., Sundbom, M., Hellström, P. M., Sellin, M. E. (2021) *Salmonella enterica* serovar Typhimurium exploits cycling through epithelial cells to colonize human and murine enteroids. *MBio*. 12(1):e02684-20.

III. **Geiser, P.**, Di Martino, M. L., Lopes, A. C. C., Bergholtz, A., Sundbom, M., Skogar, M., Graf, W., Webb, D.-L., Hellström, P. M., Eriksson, J., Sellin, M. E. (2024) Determinants of divergent *Salmonella* and *Shigella* epithelial colonization strategies resolved in human enteroids and colonoids. *Manuscript, in revision*.

IV. **Geiser, P.**, Westman, J., Ceylan, M., Bosman, W., von Beek, C., Artursson, P., Kjellén, L., Pelaseyed, T., Sellin, M. E. (2024) A naked host cell membrane is the optimal surface for targeting by *Salmonella*. *Unpublished manuscript*.

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List of additional papers not included in the thesis

Gottschlich, L., **Geiser, P.**, Bortfeld-Miller, M., Field, C. M., Vorholt, J. A. (2019) Complex general stress response regulation in *Sphingomonas melonis* Fr1 revealed by transcriptional analyses. *Scientific Reports*, 9(1):9404.

Hausmann, A., Böck, D., **Geiser, P.**, Berthold, D. L., Fattinger, S. A., Furter, M., Bouman, J. A., Barthel-Scherrer, M., Lang, C. M., Bakkeren, E., Kolinko, I., Diard, M., Bumann, D., Slack, E., Regoes, R. R., Pilhofer, M., Sellin, M. E., Hardt, W.-D. (2020). Intestinal epithelial NAIP/NLRC4 restricts systemic dissemination of the adapted pathogen S*almonella* Typhimurium due to site-specific bacterial PAMP expression. *Mucosal Immunology*, 13(3):530-544.

Fattinger, S. A., **Geiser, P.**, Samperio Ventayol, P., Di Martino, M. L., Furter, M., Felmy, B., Bakkeren, E., Hausmann, A., Barthel-Scherrer, M., Gül, E., Hardt, W.-D., Sellin, M. E. (2021). Epithelium-autonomous NAIP/NLRC4 prevents TNF-driven inflammatory destruction of the gut epithelial barrier in *Salmonella*-infected mice. *Mucosal Immunology*, 14(3):615-629.

Fattinger, S. A., Maurer, L., **Geiser, P.**, Bernard, E. M., Enz, U., Ganguillet, S., Gül, E., Kroon, S., Demarco, B., Mack, V., Furter, M., Barthel, M., Pelczar, P., Shao, F., Broz, P., Sellin, M. E., Hardt, W.-D. (2023) Gasdermin D is the only Gasdermin that provides protection against acute *Salmonella* gut infection in mice. *Proc Natl Acad Sci U S A*, 120(48):e2315503120.

Geiser, P., van Rijn, J. M., Sellin, M. E. (2023) Time-lapse imaging of inflammasome-dependent cell death and extrusion in enteroid-derived intestinal epithelial monolayers. *Methods in Molecular Biology*, 2641:203-221.

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Thesis defense

This thesis will be presented and publicly defended on 21st of November 2024 at 9.00 am, in room A1:111a at the Biomedical Centre (BMC) of Uppsala University in Uppsala, Sweden.

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Contents

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AHL	Acyl homoserine lactone
AIEC	Adherent invasive *Escherichia coli*
AIM2	Absent in melanoma 2
ARNO	ARF nucleotide binding site opener
ATP	Adenosine triphosphate
BCV	Bacterium-containing vacuole
BMP	Bone morphogenetic protein
DAMP	Danger-associated molecular pattern
DNA	Deoxyribonucleic acid
e.g.	Example given
EAEC	Enteroaggregative *Escherichia coli*
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extraintestinal pathogenic *Escherichia coli*
FAE	Follicle-associated epithelium
Fuc	Fucose
GAG	Glycosaminoglycan
GBP	Guanylate-binding protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GlcNAc	N-Acetylglucosamine
GP2	Glycoprotein-2
GSDMD	Gasdermin D
GTP	Guanosine triphosphate
H-NS	Heat-stable nucleoid-structuring protein
HS	Heparan sulfate
i.e.	id est
IEC	Intestinal epithelial cell

IL	Interleukin
ISC	Intestinal stem cell
LPS	Lipopolysaccharide
M cell	Microfold cell
MDCK	Madin-Darby Canine Kidney
MUC	Mucin
N-WASP	Neural Wiskott Aldrich Syndrome Protein
NAIP	NLR apoptosis inhibitory protein
NLR	Nucleotide-binding oligomerization domain, leucine-rich repeat
NLRC4	NLR family caspase activation and recruitment domain-containing 4
NLRP	NLR family pyrin domain-containing
nm	Nanometer
NSS	Near-surface swimming
NT	N-terminal domain
OMP	Outer membrane protein
p.i.	Post infection
PAMP	Pathogen-associated molecular pattern
pH	Potential of hydrogen
PMN	Polymorphonuclear cell
PRR	Pattern recognition receptor
RANKL	Receptor activator of NF-κB ligand
S.Tm	*Salmonella enterica* serovar Typhimurium
SCC	*Salmonella*-containing compartment
SCV	*Salmonella*-containing vacuole
SGEF	Src homology domain 3-containing GEF
Sia	Sialic acid(s)
SPI	*Salmonella* pathogenicity island
spp.	Species
T1SS	Type One Secretion System
T3SS	Type Three Secretion System
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
wt	Wildtype

Introduction

Structure and function of the intestinal epithelium

The intestine is a remarkable organ combining essential absorptive and barrier functions in seeming conflict with each other at first glance. As it provides an increased surface area for efficient nutrient uptake and water resorption, the intestinal epithelium constitutes one of the largest interfaces between the body and the environment (1). However, while being selectively permeable to such small, food-derived molecules, the intestinal epithelium simultaneously fulfills a critical barrier function restricting microbial access to underlying and systemic tissues. The intestine is lined by a single layer of polarized intestinal epithelial cells (IECs) separating the luminal content from the lamina propria and deeper tissues. Structurally, the epithelium is divided into crypts, invaginations into the lamina propria, and villi, protrusions that dramatically enlarge the epithelial surface to ensure efficient nutrient uptake in the small intestine (Figure 1). In the colon, on the other hand, a flat epithelial surface protects the intercrypt epithelium from damage by mechanical shearing (2).

The entire epithelium is renewed every three to five days by the remarkable proliferative capacity of intestinal stem cells (ISCs) residing at the crypt bottom. ISCs divide asymmetrically to give rise to transit amplifying cells, which in turn replenish the specialized absorptive and secretory IEC populations. As they migrate upwards along the crypt-villus axis, IECs terminally differentiate and eventually undergo spontaneous apoptosis to be sloughed from the epithelium at the villus tip (2). While absorptive enterocytes and colonocytes responsible for nutrient uptake and water absorption represent the majority of the IEC population, different subsets of more sparsely distributed secretory cells perform a range of crucial complementary functions. Goblet cells secrete soluble mucins such as MUC2 that form a protective and selectively permeable mucus layer to separate the epithelium from luminal microbes (3, 4). Enteroendocrine cells produce peptide hormones that regulate digestive functions (5). Tuft cells secrete the cytokine interleukin-25 (IL-25) and play an important role in the defense against parasitic helminth infections (6). Paneth cells are a unique subset of secretory cells primarily found in the small intestine. They migrate towards the crypt bottom upon differentiation, where they fulfill important functions for maintenance of the stem cell niche and antimicrobial defense via the secretion of various antimicrobial molecules (7, 8). These antimicrobials are retained in the mucus layer to enhance its

biochemical barrier effect (9). Furthermore, the intestinal epithelial barrier is decorated with a cell surface-attached layer of glycoproteins collectively termed the glycocalyx that among others comprises the transmembrane mucins MUC1, MUC13 and MUC17 expressed in the enterocyte/colonocyte apical brush border (Figure 2) (10).
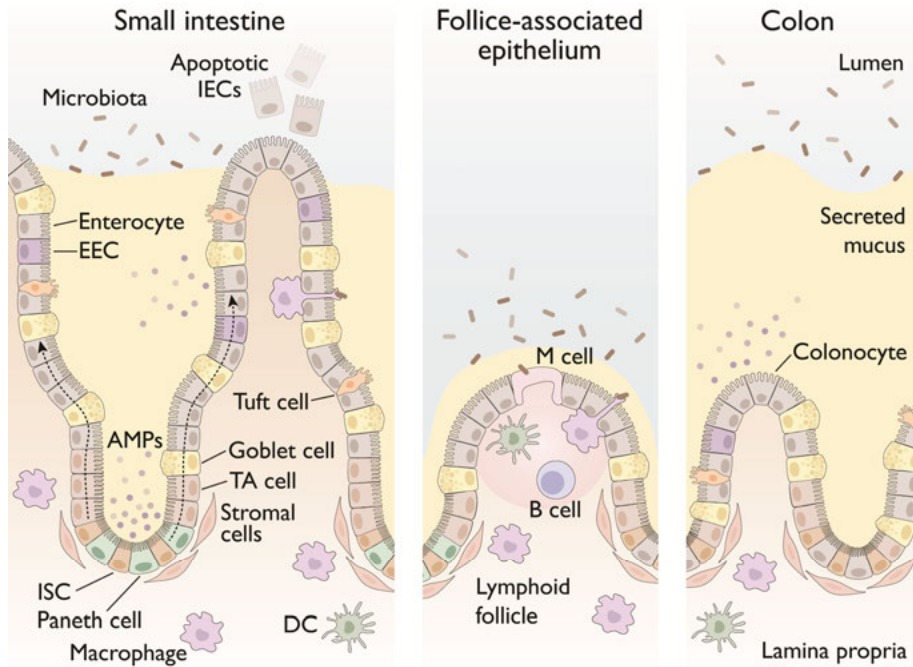

Figure 1. Structure of the intestinal epithelium. The intestinal epithelium consists of a single layer of polarized intestinal epithelial cells (IECs) that separates the lumen from the underlying lamina propria with resident immune cells such as dendritic cells (DCs) and macrophages. In the small intestine, the epithelium comprises crypts, invaginations into the underlying tissue, and villi, protrusions into the lumen. A flat intercrypt epithelium in the colon prevents damage resulting from shear stress by luminal contents. Constant renewal of the epithelium is ensured by intestinal stem cells (ISCs) located at the crypt bottom. ISCs give rise to a population of transit amplifying (TA) cells, which in turn repopulate the absorptive and secretory IEC lineages. Upon differentiation, IECs migrate upwards along the crypt-villus axis, until they eventually undergo apoptosis and are shed into the lumen at the villus tip. Absorptive IECs (enterocytes and colonocytes) are responsible for nutrient and water uptake. Secretory lineages comprise mucus-secreting goblet cells, hormone-producing enteroendocrine cells (EEC), mechanosensory tuft cells and crypt-resident Paneth cells. Paneth cells intercalate with ISCs and produce growth factors that shape the stem cell niche, as well as antimicrobial peptides (AMPs). Certain AMPs are also secreted by enterocytes and colonocytes. The follicle-associated epithelium features a reduced mucus layer for enhanced contact with luminal contents. M cells transcytose luminal microbes and antigens for presentation to immune cells in underlying lymphoid follicles.

As the intestinal lumen is populated by a dense and complex community of microbes, collectively termed the microbiota, the intestinal epithelium plays a key role in distinguishing commensal from pathogenic microbes. Strategically located at the environmental interface, IECs furthermore guide the development of the intestinal mucosal immune system and function as an early alert system to coordinate immune responses against pathogenic onslaught (11). While epithelial-intrinsic immune mechanisms (discussed in detail below) are pivotal in this regard, IECs also need to communicate with professional immune cells in the underlying lamina propria. Microfold (M) cells are specialized in uptake and transcytosis of luminal antigens and whole microbes, and are specifically found in the follicle-associated epithelium (FAE) overlaying immune hubs referred to as Peyer's patches or lymphoid follicles. Featuring a thinner mucus layer and reduced concentrations of Paneth cell-derived antimicrobials, luminal contact is enhanced at the FAE (12).

Intestinal colonization by pathogenic enterobacteria

Foodborne enterobacterial pathogens are ingested along with contaminated food or water and need to overcome the various physical and biochemical barriers of the intestine to colonize the lumen and epithelium. Enteropathogenic infections are a leading cause of foodborne disease all around the globe and result in a considerable burden on healthcare and agricultural systems. The most prominent causative agents of such diseases are enterobacterial species as for example *Salmonella enterica*, *Shigella* spp. and pathogenic *Escherichia coli*, noroviruses as well as protozoan parasites such as *Cryptosporidium* and *Giardia* (13). Importantly, *S. enterica* and *Shigella* spp., alongside rotaviruses, are the leading causes of deaths due to diarrheal disease, and *Shigella* in particular accounts for high numbers of deaths among young children (14, 15).

To reach the intestinal environment, enterobacterial pathogens need to survive the adverse conditions encountered in the stomach. While intestinal colonization by commensal microbiota has beneficial consequences for the host, enteropathogens bind to and/or invade the gut epithelium to elicit inflammation and disrupt the microbiota-host equilibrium. Thereby, the establishment of an extracellular niche via intimate contact with the IEC apical surface (extracellular pathogens such as enteroaggregative *E. coli*, EAEC), or an intraepithelial cytosolic or vacuolar niche (invasive/intracellular pathogens such as *Shigella* and *Salmonella*) is a key step for prolonged colonization of the host. Towards luminal colonization, pathogenic enterobacteria need to compete with the resident microbiota for available nutrient niches. Furthermore, commensals also contribute to intestinal barrier function by directly inhibiting the growth of intruders. The collective barrier effect of these microbiota-dependent processes is termed colonization resistance and represents the first hurdle to be overcome by enterobacterial pathogens (16).

Bacterial virulence factors are required for every step of epithelial colonization and suppression or activation of immune responses, but their expression comes at a fitness cost. Hence, induction needs to be tightly controlled and depends on a combination of biochemical stimuli from the host environment such as temperature, oxygen levels, osmolarity, pH, and pathogen population density (17–20). To establish an epithelial niche, enterobacterial pathogens first need to penetrate the mucus layer. Thereby, secreted mucus serves as one such cue that appears to regulate virulence gene expression and functions as a chemoattractant guiding the bacteria towards the epithelium. The important barrier function of the mucus layer is highlighted by the variety of enteropathogenic strategies to overcome it, ranging from flagellar motility to mucus-degrading enzymes and toxins, and specific targeting of regions with a reduced mucus layer such as the FAE or the murine cecum (10).

Similar to the secreted mucus layer, transmembrane mucins of the glycocalyx form an additional protective layer impermeable to bacteria-sized particles and commensals (21–23). Like their soluble counterpart, these membrane-anchored mucins are extensively O-glycosylated (Figure 2). While keeping commensals at bay, glycan moieties of transmembrane mucins and other glycoproteins also function as ligands for bacterial adhesins to promote contact with the IEC surface (24–27). The function of glycocalyx components as barrier or adhesin ligands during bacterial invasion is further studied in **Paper IV**. Once they reach the epithelium, enteropathogens may manipulate the apical surface to establish an extracellular niche, or engage in IEC invasion by exploiting host cell surface receptors such as integrins for internalization, or translocating effector proteins into the host cell to trigger bacterial uptake.
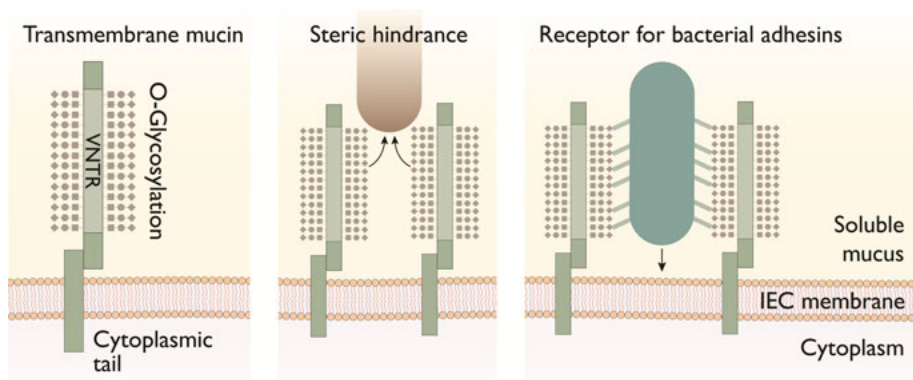
Figure 2. Transmembrane mucins of the intestinal epithelium. Transmembrane mucins such as MUC1, MUC13 and MUC17 are an important constituent of the IEC glycocalyx. These glycoproteins consist of a short cytoplasmic tail and a transmembrane domain non-covalently linked to the cleaved extracellular domain with a variable number of tandem repeats (VNTR) that are heavily O-glycosylated. Transmembrane mucins and their glycans may serve as both steric barrier towards commensal and pathogenic microbes or receptors for bacterial adhesins.

Within host cells, bacteria may inhabit a vacuolar niche by manipulating the endocytic pathway to prevent lysosomal degradation and establish a specialized pathogen-containing vacuole, or escape the endocytic vacuole to adapt a cytosolic lifestyle. While the host cell cytosol offers a nutrient-rich environment for bacterial replication, this comes at the expense of unsheltered exposure to cytosolic innate immune mechanisms such as inflammasomes or autophagy (28). Toll-like receptors expressed at the apical IEC surface are constantly exposed to commensal bacterial products and generally induce a tolerogenic response (29). In contrast, cytosolic sensors detecting intracellular pathogen-associated molecular patterns (PAMPs) alert the immune system of an intruding pathogen and downstream responses are highly pro-inflammatory. These epithelial innate immune responses aim to prevent breaching of the epithelial barrier, bacterial release into the lamina propria, and ultimately spread to systemic organs (11).

Salmonella Typhimurium infection and virulence factors

Different species of the *Salmonella* genus are associated with both typhoid fever, a systemic disease caused by *S. enterica* serovars Typhi and Paratyphi, and diarrheal disease. Infections with non-typhoidal *Salmonella* serovars Enteritidis or the prototype strain *S. enterica* serovar Typhimurium (*S.*Tm) are generally self-limiting in immunocompetent human hosts and associated with gastroenteric symptoms (30). In immunocompromised individuals, however, *S.*Tm can cause life-threatening systemic disease (31), and in mice the infection is characterized by typhoidal manifestations, hence the name Typhimurium (30). In contrast to typhoidal strains, *S.*Tm has a broad host range and can infect the intestine of various warm-blooded animals, targeting the distal small intestine and proximal colon in humans, and the cecum in mice (32–34). Low oxygen levels, high osmolarity, changes in pH and other cues associated with the intestinal environment, along with nutrient limitation resulting from increasing luminal population densities cause a bacterial subpopulation to upregulate their invasive machinery encoded on *Salmonella* pathogenicity island-1 (SPI-1) (17, 19, 35). Importantly, only a small fraction of the luminal population engages in epithelial invasion, but the induction of an inflammatory response by these intraepithelial pioneers is sufficient to provide the luminal bulk of the population with a selective advantage over the microbial community (36–38).

Flagellar motility and chemotaxis

Expression of SPI-1-encoded virulence factors is induced by the master regulator HilD, that also co-regulates flagellar motility via the flagellar master regulator FlhDC along with other motility-inducing signals (39). Consequently, HilD allows for coordinated expression of the flagellar apparatus and chemotaxis receptors, on one hand, and the syringe-like Type Three Secretion System-1 (T3SS-1) encoded on SPI-1 and its effector proteins required for epithelial cell invasion, on the other hand. Chemotaxis and flagellar motility are required for directed bacterial swimming towards the intestinal epithelium (40, 41). Furthermore, HilD has been shown to lock bacteria in a 'smooth' swimming state, which allows for straighter movements to quickly reach the epithelial surface (42). Flagellar motility and chemotaxis even play a crucial role in T3SS-1-independent invasion of Peyer's patches in the mouse ileum, which corroborates their crucial function in host cell targeting (40). Upon reaching the intestinal surface, *S*.Tm moves along the mucus-covered epithelium, a phenomenon termed near-surface swimming (NSS; (43)). Flagellar motility allows the bacterium to penetrate the protective mucus layer to reach the underlying target IECs (44). In the murine model, *S*.Tm was furthermore shown to preferentially target the cecum, which lacks a continuous mucus layer (44). Most cultured cell line models are defective for mucus secretion, and hence NSS allows the bacteria to efficiently scan the epithelial surface for suitable binding sites (43). On their swimming paths, bacteria transiently stop their movement, which preferably occurs at topological obstacles such as rounded up mitotic cells or cell-cell junctions (43).

Adhesion

Transient stopping along NSS paths allows for reversible binding to the epithelial surface via an extensive repertoire of adhesins expressed by *S*.Tm, whereas insertion of the translocon at the T3SS-1 tip into the host cell plasma membrane has been suggested to mediate irreversible docking (Figure 3) (45, 46). These fimbrial and non-fimbrial adhesins are expected to play a role for infection of various cell types and host species and may contribute to the broad host range of *S*.Tm. Hence, only a subset of adhesins is expressed under standard laboratory conditions, while other adhesins are induced under specific environmental conditions or time points during infection and require inducible expression to study their structure and adhesive properties (47). While many adhesins bind glycan-based ligands of the glycocalyx, others bind cell surface receptors such as integrins to directly mediate invasion via a zipper-like, T3SS-1-independent mechanism (48), and yet others have completely unknown ligand specificities.
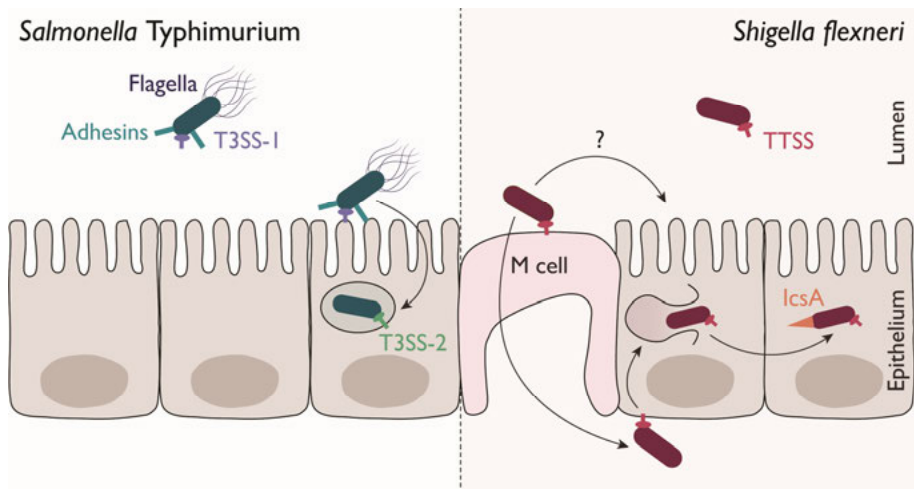
Figure 3. Colonization of the intestinal epithelium by *S.*Tm and *S. flexneri*. *S.*Tm employs flagellar motility to reach the epithelial surface and is equipped with an extensive repertoire of adhesins as well as a syringe-like T3SS-1 to inject a cocktail of virulence factors into the host cell and trigger its uptake. Within IECs, *S.*Tm adopts a primarily vacuolar lifestyle and shapes its intracellular niche via a second T3SS (T3SS-2). *S. flexneri* is non-motile, but also employs a T3SS for host cell invasion and establishment of its intracellular niche. According to the classical paradigm *S. flexneri* crosses the epithelium via M cell transcytosis and subsequently invades IECs from the basolateral side. To what extent direct apical invasion contributes to epithelial colonization remains unclear. Within host cells, *S. flexneri* promptly lyses the endocytic vacuole, adopts a cytosolic lifestyle and polymerizes host actin via the actin nucleator IcsA to spread to neighboring IECs by actin-based motility.

Fimbrial adhesins

So far, the binding specificity for three fimbrial adhesins has been described, and all were found to bind glycan-based ligands in a lectin-like manner. Fim is the best characterized fimbrial system and comprises the FimH fimbrial tip that determines the broader binding specificity for mannose, as well as a shaft formed by FimA structural subunits that may further modulate the fine glycan specificity (49). FimH binds Glycoprotein-2 (GP2) exclusively expressed on the apical surface of intestinal M cells in a mannose-dependent manner, which was suggested to mediate bacterial transcytosis and subsequent mounting of adaptive immunity (24). Two other fimbrial adhesin systems, Std and plasmid-encoded fimbriae (Pef) are not expressed under standard laboratory conditions. Induction of expression by the deletion of a negative transcriptional regulator or ectopic expression in *E. coli*, respectively, has allowed to study their binding specificities ((50, 51); Table 1).

The SPI-4-encoded SiiE adhesin system

Another well-studied adhesin is SiiE encoded on SPI-4 along with its cognate Type One Secretion System (T1SS) required for secretion. It was initially

identified to mediate apical adhesion to and invasion of polarized Madin-Darby canine kidney (MDCK) epithelial cells while being dispensable in infection of the commonly used cervical carcinoma cell line HeLa (52). Furthermore, SPI-4 cooperates with SPI-1 to breach the epithelial barrier and initiate gastrointestinal inflammation in a model of murine colitis (53). A follow-up study identified the Immunoglobulin repeats of SiiE to mediate multiple interactions with host cell ligands and to thereby bind sialic acid and N-acetylglucosamine (GlcNAc) moieties in a lectin-like manner ((54); Table 1). The authors of the latter study hypothesized that the apical brush border glycocalyx specific to polarized cells might form a barrier that prevents insertion of the T3SS-1 translocon into the host cell membrane, which in turn might be overcome by such lectin-like interactions of SiiE with brush border glycoproteins to allow for effector translocation and bacterial invasion. Indeed, later studies found SiiE to bind common intestinal transmembrane mucins MUC1 (26) and MUC13 (55). While MUC1 promoted invasion of the colorectal adenocarcinoma cell line HT29-MTX in an SiiE- and sialic acid-dependent manner (26), MUC13 was found to play a protective role in a murine enterocolitis model by acting as a releasable decoy receptor shed from the epithelial surface upon *S.*Tm binding (55). The role of SiiE during epithelial colonization and its interaction with the intestinal glycocalyx and MUC1 in particular are further discussed in **Papers III** and **IV**.

Other adhesins and adhesive cell surface structures

While absent under standard laboratory conditions, expression of the autotransported adhesins MisL and ShdA during infection of the murine cecum has been suggested to promote prolonged colonization of the host via binding to the extracellular matrix component fibronectin ((56–58); Table 1). Furthermore, OmpV, an outer membrane protein, also mediates binding to fibronectin and α1β1-integrin to induce actin rearrangements and enhance invasion of sub-confluent layers of the colorectal adenocarcinoma cell line Caco-2 from the basolateral side (59). Another outer membrane protein PagN (*phoP*-activated gene N) has been described as an adhesin and invasin that mediates T3SS-1-independent invasion via binding to heparan sulfate (HS) proteoglycans functioning as a co-receptor for β1-integrin-mediated internalization (25, 48, 60). In a similar manner, Rck (Resistance to complement killing) binding to epidermal growth factor receptor (EGFR) was found to mediate both adhesion and invasion under some conditions (61, 62). Finally, also cell surface appendages with other primary functions have been implied in adhesion to the host cell surface. *S.*Tm lipopolysaccharide (LPS), a major component of the Gram-negative outer membrane, has been proposed to bind multivalently to host cell glycans (63). Flagella have been reported to interact with the host cell plasma membrane (64, 65), and the T3SS-1 translocon proteins SipB and SipC bind membrane cholesterol (66) and polymerized actin (67), respectively, to mediate intimate host cell attachment (46, 68).

Table 1. Overview of *S*.Tm adhesins with characterized ligand specificities.

Adhesin	Type	Expressed under standard conditions	Ligand specificity	References
FimH	fimbrial	yes	mannose, GP2	(24)
Std	fimbrial	no	terminal Fucα1-2	(51)
Pef	fimbrial	no	Lewis X	(50)
SiiE	T1SS-secreted	yes	Sia, GlcNAc, MUC1, MUC13	(26, 55)
ShdA	autotransporter	no	fibronectin	(57, 58)
MisL	autotransporter	no	fibronectin	(56)
OmpV	OMP	yes	fibronectin, α1β1-integrin	(59)
PagN	OMP/invasin	no	HS GAGs, β1-integrin	(25, 48, 60)
Rck	OMP/invasin	no	EGFR	(61, 62)

OMP, outer membrane protein; GP2, Glycoprotein-2; Fuc, fucose; Sia, sialic acids; GlcNAc, N-acetylglucosamine; HS, heparan sulfate; GAG, glycosaminoglycan; EGFR, epidermal growth factor receptor.

Epithelial invasion

Successful and prolonged colonization of the host as well as induction of inflammation requires *S*.Tm to invade the intestinal epithelium. The syringe-like T3SS-1 encoded on SPI-1 and its effectors comprise the primary virulence machinery for IEC invasion from the luminal face of the epithelium (Figure 3). However, T3SS-1-independent mechanisms for invasion and breaching of the epithelial barrier have been described too, and *S*.Tm most likely causes gastroenteritis via the combined action of all these pathways (69–71).

T3SS-1-mediated host cell invasion

T3SSs consist of over 20 proteins and are evolutionarily related to the bacterial flagellum. The needle complex mediates passage of secreted effector proteins through the bacterial envelope and comprises a multi-ring base, a needle-like projection, in the case of *S*.Tm composed of the molecular building block PrgI, and an inner rod, in *S*.Tm formed by PrgJ subunits, that connects the needle to the base (72). Mutants deficient for *invG*, encoding a component of the outer ring of the base, lack a structural T3SS-1 and are non-invasive (73). The translocator proteins SipB, SipC and SipD are required for insertion of the needle tip, formed by SipD, into the host cell membrane by forming a trans-locon pore. This pore comprises SipB and SipC and forms a channel to allow for effector delivery into the host cell cytosol (68, 74).

The translocated effectors form a complex regulatory network to modulate host cell signaling and actin polymerization and trigger bacterial uptake. SopE and SopE2 act as guanine nucleotide exchange factors (GEFs) activating Rho

GTPases to initiate actin polymerization at the bacterial entry site (75, 76). These Rho GTPases act as molecular switches by cycling between a GDP-bound, inactive and a GTP-bound, active conformation that binds downstream interaction partners. Thereby, cellular GEFs accelerate the replacement of GDP for GTP to stabilize the active state, whereas their counterplayers, GTPase-activating proteins, promote GTP hydrolysis to GDP and favor the inactive conformation (77). SopE and SopE2 mimic such host GEFs and directly activate the Rho GTPases Rac1 and Cdc42 for local actin polymerization via the nucleation-promoting factor N-WASP (Neural Wiskott Aldrich Syndrome Protein) and the actin nucleator Arp2/3 (75, 76). SopB, another T3SS-1 effector, indirectly stimulates the endogenous GEFs SGEF (Src homology domain 3-contining GEF) (78) and ARNO (ARF nucleotide binding site opener) (79) by acting as a phosphoinositide phosphatase that alters the membrane phospholipid composition at the entry site (80, 81). This change in phosphoinositide composition activates Arf GTPases that cooperate with the above-mentioned Rho GTPases to initiate actin polymerization (79). In addition, SopB is involved in the mobilization of membrane reservoirs for efficient *S*.Tm internalization (82), and SopB-mediated changes in phosphoinositide pools have also been implicated in membrane invagination and fission during bacterial uptake into an endocytic vacuole (83). Collectively, SopB, SopE and SopE2 are the main drivers of invasion in cultured cell lines and mediate extensive actin remodeling to form large entry structures referred to as membrane ruffles. While cell lines provide a valuable model to disentangle the overlapping molecular functions of individual T3SS-1 effectors, the effector requirements for invasion of human and murine primary IECs differ markedly with the largest contribution of another effector, SipA, instead of the above-mentioned ruffle inducers (84–86). SipA reduces the critical concentration for actin polymerization and directly binds to polymerized actin to increase its stability and prevent depolymerization and severing (87, 88). Furthermore, SipA cooperates with SipC to promote nucleation and bundling of polymerized actin by the latter (89).

T3SS-1-independent, Zipper-like host cell invasion
While Trigger-like entry via the T3SS-1 and its effectors clearly is the main driver of epithelial cell invasion, alternative Zipper-like entry mechanisms have been identified in cultured cell lines, but to what extent they contribute to epithelial colonization *in vivo* is unclear (71). The *Salmonella* invasins Rck and PagN bind to host cell surface receptors to induce downstream signaling via the phosphoinositide 3-kinase pathway and mediate actin rearrangements via the Rho GTPase Rac1 (48, 60–62, 90). *pagN* expression is induced intracellularly via the PhoPQ two-component regulatory system, an important global virulence regulator in *Salmonella* (*pag* = PhoP-activated gene) (91). Therefore, it has been hypothesized that its expression might prime intracellular bacteria for host cell reinvasion upon bacterial release (60). *rck*

expression, on the other hand, is induced by the quorum sensing regulator SdiA in response to acyl homoserine lactone (AHL) (92). Interestingly, *Salmonella* is deficient for AHL production, but Rck may be expressed in response to AHL produced by other bacterial species in the intestinal lumen (93). Finally, even *S*.Tm deficient for all three known invasion machineries, namely the T3SS-1, Rck and PagN, has been shown to retain some invasiveness via a Zipper-like mechanism in certain cell lines (94).

Passive mechanisms for transepithelial translocation

In addition to these active mechanisms of IEC invasion and subsequent transcellular transport, *S*.Tm can also breach the epithelial barrier and induce inflammation via a passive, SPI-1-independent pathway (69). Thereby, T3SS-1-deficient *S*.Tm Δ*invG* translocate across the epithelium via luminal sampling by dendritic cells of the lamina propria (70), transcytosis via FAE-associated or villous M cells (95, 96) or potentially paracellular transport. Induction of colitis is dependent on a second T3SS-2 encoded on SPI-2 and expressed intracellularly (69). During infection with wildtype (wt) *S*.Tm, the active and passive pathways cooperate to achieve efficient colonization and full-blown inflammation. Interestingly, *S*.Tm has also been shown to induce trans-differentiation of follicle-associated IECs into M cells via the effector SopB to promote translocation via the passive pathway (97), further highlighting the importance of crosstalk between various entry mechanisms. Such an array of invasive strategies provides access to a variety of host cell types during the course of the infection and might even contribute to the broad host range of *Salmonella*.

Intraepithelial lifestyle

Establishment of a vacuolar intracellular niche

Following T3SS-1-mediated invasion, which is the main entry pathway in epithelial cells, *S*.Tm is initially contained within a tight vacuole termed the *Salmonella* containing compartment (SCC) (98). The effectors SopB and SopD cooperatively promote SCC fission from the plasma membrane and the formation of invasion-associated macropinosomes (99). Through fusion with these surrounding macropinosomes generated during the entry process, the SCC enlarges and progressively matures into an intracellular niche permissive for bacterial replication and referred to as the *Salmonella*-containing vacuole (SCV; Figure 3) (98). Via fusion with infection-associated macropinosomes and formation of vacuole-associated tubules, the SCV growth is regulated dynamically in a SopB-dependent manner (100, 101). During its maturation, the SCV migrates towards the microtubule-organizing center, where *S*.Tm vacuolar replication is initiated (102).

Within the SCV, the SPI-1-encoded T3SS-1 and its effectors are downregulated, while expression of a second T3SS-2 encoded by SPI-2 is induced (103). Importantly, effectors of both T3SSs are involved in establishment and maintenance of the intracellular vacuolar niche (71). Besides its multiple functions during *S*.Tm invasion, the T3SS-1 effector SopB has been shown to phosphorylate and activate the pro-survival kinase Akt (104) to suppress apoptosis in epithelial cell lines and prolong the time for intracellular vacuolar (and cytosolic) niche establishment (105). SPI-2 expression is induced inside epithelial and non-epithelial host cells (106, 107) via the SPI-2-encoded SsrAB two-component system in response to signals specific to the vacuolar environment such as low magnesium or calcium levels and phosphate starvation (108). During SCV maturation, secretion of T3SS-2 effectors is induced by vacuole acidification (109). These effectors inhibit phagolysosomal fusion of the SCV (110) and promote intracellular survival and proliferation (110–113). Furthermore, the T3SS-2 is required for *S*.Tm intraepithelial traversal and basal exit to the lamina propria, where they are taken up by phagocytes (114, 115) and initiate systemic spread to the draining mesenteric lymph nodes (116). This is in line with a key role of the T3SS-2 during systemic infection in mice, as SPI-2-deficient mutants are avirulent in these models (106, 113, 117).

Establishment of a cytosolic intracellular niche

Transcriptome analysis of intracellular *S*.Tm within HeLa cells found that while SPI-1 and flagella were downregulated at early time points post infection (p.i.), both machineries were unexpectedly upregulated again at four to six hours p.i.. Similarly, components of glucose metabolism and glycolysis such as the *uhpT* gene encoding a glucose-6-phosphate transporter were also upregulated (103). These results have been explained by a later, striking discovery that a subpopulation of intracellular *S*.Tm escapes the endocytic vacuole and hyperreplicates in the cytosol of epithelial host cells (118). Thereby, vacuole rupture occurs early during infection and is correlated with the absence of SCC fusion with macropinosomes (98). Vacuole rupture and cytosolic replication are dependent on the T3SS-1 and its effectors SopB and SipA (119). SopB and SopF antagonistically regulate SCV membrane integrity, with SopB increasing rupture and SopF preventing it (120). Furthermore, intravacuolar expression of *pagN* may also contribute to vacuolar escape and cytosolic hyperreplication (121). Cytosolic *S*.Tm are invasion-primed, as they express the T3SS-1, flagella and the SiiE adhesin (118, 122), and deliver a second wave of SopB to the cytosol, which may prolong host cell survival (123). Access to the nutrient-rich cytosolic environment allows *S*.Tm to replicate at a much higher rate compared to the SCV, and hence the cytosolic subpopulation represents a significant proportion of the total intracellular population, even though hyperreplication only occurs in a minority (10-20%) of infected host cells (124, 125). However, the cytosolic lifestyle also exposes intracellular bacteria to antibacterial responses, namely cytosolic innate

immune receptors and autophagy that limit bacterial hyperreplication (28). In addition to the SCV and cytosolic intracellular niche, *S*.Tm has also been found to enter a non-growing, dormant state in epithelial cells, whereby the bacteria are confined within a vacuolar compartment lacking most SCV markers (126).

Interconnection between different stages of colonization

Rather than occurring strictly sequential, the different stages of *S*.Tm epithelial colonization are interconnected in various ways. In addition to their crucial role in targeting of the epithelial surface for invasion, flagellar motility and chemotaxis have been shown to contribute to luminal colonization by enabling access to spatially limited reservoirs of high-energy nutrients in the inflamed gut lumen (127). The T3SS-1 and the invasins Rck and PagN are involved in both host cell binding and internalization (46, 60, 61), and T3SS-1 and its effectors furthermore contribute to the establishment of intracellular vacuolar and cytosolic replicative niches (86, 119, 128). Persistent colonization of the luminal environment *in vivo* in mice requires both SPI-1 and SPI-2 activity (69), and intraepithelial bacteria induce an inflammatory environment that provides the luminal population with a competitive advantage over the microbiota (36, 37). In addition, a role for intracellular, cytosolic *S*.Tm in re-seeding of the luminal population via shedding of epithelial cells harboring these invasion-primed and motile bacteria has been proposed (118, 129, 130) and is further commented on in **Paper II**.

Shigella flexneri infection and virulence factors

S. flexneri represents the leading cause of shigellosis among the four described *Shigella* species in low- and middle-income countries. Besides causing endemic disease, particularly in young children below the age of five, *Shigella* is also responsible for traveler's diarrhea (131). In contrast to *Salmonella*, *Shigella* is a primate-restricted pathogen that primarily spreads via the fecal-oral route (131) and displays a strikingly low infectious dose with 10-100 bacteria being sufficient to cause disease in some cases (132). *Shigella* colonizes the terminal ileum, colon and rectum where it causes watery or bloody diarrhea with fever and abdominal cramps known as dysentery. Infections are usually self-limiting, but may occasionally spread systemically and become life-threatening in immunocompromised individuals or if adequate medical care is lacking (131).

    Shigella carries a virulence plasmid that is required for epithelial cell invasion and virulence *in vivo* (133). The majority of genes required for host cell invasion are clustered in a 31 kilobase 'entry region' on the virulence plasmid (134, 135). This 'entry region' includes the *mxi* (membrane expression of *ipa*)

and *spa* (surface presentation of *ipa*) genes, required for the formation of a structural T3SS, as well as the *ipa* (invasion plasmid antigens) and *ipg* (invasion plasmid genes) genes encoding T3SS effectors that trigger invasion and their chaperones (136). Analogous to *Salmonella*, the T3SS is required for efficient *Shigella* invasion. Mutants deficient for *mxiD*, encoding the major structural component of the outer membrane ring of the T3SS basal body, do not assemble a structural T3SS and are both non-invasive and avirulent (137). Additional T3SS effectors and virulence factors are encoded outside the 'entry region'. These include, among others, the transcriptional master regulator VirF, the actin nucleator IcsA (intercellular spread) required for cell-to-cell spread and the *osp* (outer *Shigella* proteins) and *ipaH* genes. The classes of *osp* and *ipaH* effectors generally have post-invasion functions to modulate host cell transcriptional and immune responses (136, 138).

Regulation of virulence factor expression and secretion

Expression of virulence genes is costly and reduces pathogen fitness and is therefore tightly regulated. In the case of *Shigella*, virulence gene expression is repressed at temperatures below 32°C and induced upon a shift to 37°C, the temperature encountered within the host (20). It is further modulated by additional cues of the host environment, such as pH, osmolarity and iron availability (18, 136). Mechanistically, a temperature shift to 37°C causes structural alterations of a DNA thermosensor bend to relieve repression by a general bacterial virulence repressor, H-NS (heat-stable nucleoid-structuring protein) and induce expression of the transcriptional activator VirF (139). VirF is the major transcriptional regulator of *Shigella* virulence genes and induces expression of IcsA and the transcription factor VirB, which are also repressed by H-NS at non-permissive temperatures (140, 141). VirB, in turn, controls the expression of the *mxi* and *spa* genes for formation of a structural T3SS, a set of 'early effectors' and their chaperones, i.e. the *ipa*, *ipg* and selected *osp* genes (18). Expression of a second set of 'late effectors' with intracellular functions, namely the *osp* and *ipaH* genes, is induced via the transcription factor MxiE upon T3SS activation and effector secretion (142, 143). Furthermore, effector secretion is repressed under anaerobic conditions encountered in the gut lumen. Increased oxygenation levels just above the epithelial surface provided by mucosal vascularization in turn induce T3SS-dependent effector secretion (144).

Epithelial invasion

T3SS-mediated host cell invasion

In contrast to *Salmonella*, *Shigella* is non-motile and the role for adhesins during epithelial invasion is not well established (Figure 3). Several putative

adhesins have been described, but are generally not expressed under standard laboratory culture conditions. Bile salts encountered by *S. flexneri* during gastrointestinal passage, as well as other chemical cues mimicking the *in vivo* environment have been suggested to promote adhesin expression, adhesion and invasion (145–150). As for *Salmonella*, interaction of *Shigella* LPS with host cell surface glycans may further promote adhesion (63).

IEC invasion is dependent on the T3SS and its effectors that locally remodel the actin cytoskeleton to force bacterial uptake via a similar mechanism to what was described for *S*.Tm above (Figure 3) (138). The needle tip and translocon proteins IpaB, IpaC and IpaD are required for epithelial invasion and virulence (151). Host cell contact induces prompt T3SS-mediated secretion of preformed cytoplasmic pools of IpaB, IpaC and IpaD (152, 153). A pre-invasion needle tip complex composed of IpaB and IpaD makes initial contact with the host cell plasma membrane, whereby IpaB binds cholesterol and interacts with cholesterol-enriched membrane subdomains termed lipid rafts (66, 154). Contact with the host cell membrane marks T3SS activation and leads to secretion of IpaC and subsequent insertion of an IpaB-IpaC complex into the host cell membrane to form a multimeric translocon pore (155, 156). Besides its translocon function, IpaC induces actin cytoskeletal rearrangements for bacterial uptake by activating the Rho GTPase Cdc42 (157) and the tyrosine kinase Src (158). However, in contrast to *S*.Tm SopE2, IpaC does not directly interact with Cdc42 (159). IpaC furthermore interacts with the intermediate filament vimentin, and this interaction is required for stable docking to the host cell surface and efficient effector translocation (160). Analogous to *S*.Tm SopE and SopE2, the *S. flexneri* T3SS effectors IpgB1 and IpgB2 act as GEFs for activation of the Rho GTPases Rac1, Cdc42 and RhoA to nucleate actin polymerization at the bacterial entry site (161–163). Similar to *S*.Tm SopB, *S. flexneri* IpgD acts as a phosphatidylinositol phosphatase to activate the host endogenous GEF ARNO and enhance Rac1 and Cdc42 activity for nucleation of actin polymerization (164–166). Finally, the *S. flexneri* T3SS effector IpaA binds the cytoskeletal protein vinculin to unmask an F-actin binding domain and enhance its interaction with polymerized actin (167, 168). The IpaA-vinculin complex induces actin depolymerization to avoid excessive polymerization, which would abort bacterial uptake (167). In addition, T3SS effector secretion can be artificially induced by the dye Congo red (169), which is routinely used as a simple, time- and resource-efficient screen for virulence (170).

Mechanisms providing intraepithelial access

S. flexneri invasion from the apical face of the epithelium is inherently inefficient, whereas increased invasion efficiencies have been observed from the basolateral side in both cell line and enteroid models (171–173). The reasons for this low apical invasion efficiency are not entirely understood and may seem surprising in light of the low infectious dose for *Shigella*.

Experimentally, low apical invasion efficiencies can be circumvented by centrifugation of the bacteria atop the monolayer (174), ectopic adhesin or invasin expression (175, 176) or coating of the bacteria with poly-L-lysine (177) to promote contact of the T3SS with the host cell plasma membrane. *In vivo*, interaction with host-derived factors may promote invasion via a similar coating mechanism, as human enteric α-defensin 5 has been shown to increase *S. flexneri* adhesion and invasion (178). The commonly used monolayer models furthermore lack 3D tissue architecture, and both organ-on-chip and *in vivo* models have suggested that *S. flexneri* preferentially targets colonic crypts for invasion (179, 180). Peristaltic-like movements, another host factor omitted in simplistic tissue culture models, may further promote invasion (180).

According to the classical paradigm, *Shigella* penetrates the intestinal epithelium via M cells (Figure 3) and induces lytic cell death of underlying macrophages to gain access to the basolateral side (181–184). This role of M cells during *S. flexneri* epithelial translocation and colonization has recently been confirmed in enteroid/colonoid models (173, 185). Furthermore, inflammatory mediators released during *S. flexneri* infection recruit polymorphonuclear cells (PMNs), and epithelial penetration of these immune effector cells has been indicated as an additional route for intraepithelial colonization, both *in vivo* and in the enteroid model (186, 187). Such an increase in epithelial permeability can be experimentally mimicked by EGTA treatment to disrupt tight junctions (171, 172), or a malnourished enteroid epithelium, which furthermore induces the formation of M cells (188). Finally, *S. flexneri* may even actively disrupt epithelial tight junctions to destabilize barrier integrity, gain basolateral access and promote PMN transmigration (189, 190). Mechanisms of epithelial colonization will be further discussed in **Paper III**.

Intraepithelial lifestyle

Establishment of a cytosolic intracellular niche

Like *S*.Tm, *S. flexneri* is internalized into a tight bacterium-containing vacuole (BCV) surrounded by macropinosomes (191). To replicate intracellularly, which marks a crucial step for *Shigella* pathogenesis, the bacterium needs to lyse the endocytic vacuole shortly after internalization to establish a cytosolic replicative niche (192, 193). BCV lysis and vacuolar escape requires the T3SS translocators IpaB, IpaC and IpaD (194–197) and is further enhanced by the effectors IpgD and IcsB (191, 198–200). Interestingly, differences in the *S*.Tm and *S. flexneri* translocon proteins SipC and IpaC are responsible for their distinctive intracellular lifestyles, as expression of IpaC in a *sipC*-deficient mutant promoted prompt vacuolar escape of *S*.Tm (194, 196). In contrast to *S*.Tm invasion, interaction of the BCV with macropinosomes formed during the entry process promotes efficient vacuolar escape in an IpgD-dependent manner (191, 198). As high nutrient availability of the cytosolic environment comes

with increased exposure to innate immune receptors (28), *S. flexneri* secreted effectors also suppress various innate immune and cell death mechanisms or activate pro-survival pathways (201–203). These T3SS effectors are generally part of the second wave of MxiE-dependent effectors (138) and will be discussed in more detail in a following section on inflammasomes of the intestinal epithelium.

Intraepithelial lateral spread

IcsA (intracellular spread)-mediated polymerization of host actin is necessary and sufficient for *S. flexneri* cell-to-cell spread (204–207). Cell-to-cell spread represents another crucial step during *S. flexneri* pathogenesis (Figure 3), as *icsA*-deficient mutants are attenuated in different infection models and in humans (182, 204, 207–210). IcsA is encoded outside the 'entry region' on the virulence plasmid and expressed under the control of VirF (18). Its secretion is independent of the T3SS and it localizes to the posterior pole of the bacteria, where it induces actin polymerization (211). Mechanistically, IcsA mimics Cdc42 as it binds to and activates N-WASP to form a ternary complex with the actin nucleator Arp2/3. IcsA furthermore enhances interaction of the N-WASP N-terminal domain with filamentous actin to stabilize the filaments and attach them to the bacterial surface (212).

Once *S. flexneri* reaches the plasma membrane, actin-based motility leads to the formation of membrane-bound protrusions that are endocytosed by neighboring cells. Dissolution of the double-membrane vacuole formed in this process leads to bacterial release into the cytosol of the recipient cell and initiates a new cycle of replication and lateral spread (213, 214). While T3SS activity is downregulated upon BCV escape shortly after internalization, it is reactivated, presumably by membrane contact, in protrusions formed during cell-to-cell spread (215). Importantly, the T3SS, its translocators IpaB, IpaC and IpaD, and several of its effectors are required for efficient protrusion formation (216), resolution of protrusions into double-membrane vacuoles (217, 218) and lysis of these secondary vacuoles (217, 219–223), all of which are important for *S. flexneri* virulence. Finally, IcsA-mediated cell-to-cell spread has also been observed to occur from M cells directly to neighboring IECs, providing an alternative scenario to the classical paradigm of basolateral release and subsequent reinvasion of the epithelium (224).

Inflammasomes of the intestinal epithelium

Inflammasomes were initially discovered in innate immune cells as cytosolic multiprotein signaling complexes that activate inflammatory caspases (225). Catalytic autoprocessing of the caspase in the inflammasome complex results in its activation and downstream proteolytic cleavage of pro-IL-1β and pro-IL-18. Besides secretion of mature forms of these pro-inflammatory

cytokines, induction of an inflammatory mode of cell death termed pyroptosis is another crucial downstream consequence of inflammasome signaling (226). Since the initial discovery, the activation mechanisms for different inflammasome types and common downstream responses have been characterized extensively, with a major focus on their function in innate immune cells and macrophages in particular (226). However, inflammasome components also display high expression levels in the intestinal epithelium (227–230). Due to their strategic location at the host-environmental interface, these epithelial inflammasomes have been found to play a pivotal role in early defense against invading enteropathogens (227, 231–237). The NAIP (nucleotide-binding oligomerization domain, leucine-rich repeat (NLR) apoptosis inhibitory protein)/NLRC4 (NLR family caspase activation and recruitment domain-containing 4) canonical and caspase-4/11 non-canonical inflammasomes play a major role during *S*.Tm and *S. flexneri* infection of human and murine intestinal epithelia (208, 227, 231, 232, 234, 236, 238–242) and will be discussed in detail below. In addition, epithelium-intrinsic NLRP6 (NLR family pyrin domain-containing 6) is involved in parasite control (235) and NLRP9B restricts rotavirus infection of the intestinal epithelium (237).

Inflammasome activation

Inflammasomes are activated by cytosolic pattern recognition receptors (PRRs) that function as sensors for cytosolic PAMPs and DAMPs (danger-associated molecular patterns). Ligand binding by the PRR relieves autoinhibition to induce oligomerization and recruitment of additional components of the multimeric complex (226). In contrast to Toll-like receptors (TLRs) on the IEC apical surface, PAMP detection by inflammasome PRRs allows specific detection of cytosolic microbes or microbial products as an early alert system against invasive pathogens and intracellular pathogenic activity (11, 243). Inflammasome sensors comprise members of the NLR protein family as well as AIM2 (absent in melanoma 2), pyrin and caspases-4, -5 and -11 (226). Recruitment of pro-caspase-1 to a multimeric inflammasome complex leads to its activation via proximity-induced autoprocessing (226). In non-canonical inflammasomes, on the other hand, caspase-4, -5 or -11 directly binds cytosolic LPS and features combined sensor PRR and executor caspase functions (232, 244–251).

Inflammasome-mediated pyroptotic cell death and IEC extrusion

One key downstream effector function of inflammasomes is the induction of pyroptosis, a programmed lytic and pro-inflammatory mode of cell death with release of the cytoplasmic content (226). Thereby, activated pro-inflammatory caspase-1, -4, -5 or -11 proteolytically cleave Gasdermin D (GSDMD) in its flexible linker domain to relieve structural autoinhibition by the C-terminal repressor domain and free the N-terminal (GSDMD-NT) cytotoxic domain (252–254). Subsequently, the GSDMD-NT interacts with phospholipids of the

plasma membrane inner leaflet and assembles into a multimeric pore with a diameter of 10-20 nm (255–258). Final plasma membrane rupture associated with leakage of cellular contents and DAMP release is induced by the formation of larger membrane pores by Ninjurin 1 (259).

In the intestinal epithelium, activation of IEC-intrinsic inflammasomes in response to enterobacterial pathogens induces prompt extrusion of infected IECs to restrict epithelial colonization (232–234, 236). This extrusion process may cause transient loss in barrier integrity before neighboring IECs manage to seal the gap left by the extruding cell (260). This might be exploited by enterobacterial pathogens such as *Salmonella* or *Shigella* for epithelial (re-)invasion. However, loss of barrier integrity is efficiently counteracted by a collective epithelial contraction response described in **Paper I**. Importantly, pyroptosis and IEC extrusion are two independent processes, as extrusion but not pyroptosis still occur in the absence of GSDMD (261), but the relative timing of these processes is key to ensure efficient extrusion and maintenance of barrier integrity. Indeed, in murine IECs, caspase-7 has been shown to delay pore-mediated lysis by inducing membrane repair mechanisms to preserve IEC integrity long enough to allow for completion of the extrusion process. While this capase-7-dependent mechanism is dispensable for reducing intraepithelial *S*.Tm loads, it is important for maintenance of barrier integrity to prevent intestinal damage and excessive inflammation (262). In the absence of caspase-1, canonical inflammasomes may activate apoptotic caspase-8 as a backup alternative mode of cell death, which also results in IEC extrusion, but retains cytokines and other pro-inflammatory mediators intracellularly (234, 238, 263, 264). In the absence of both inflammasome-mediated pyroptosis and backup apoptosis, programmed necrotic cell death, necroptosis, may serve as a third mechanism to ensure elimination of infected cells (265, 266), which may however induce excessive inflammation and eventually loss of barrier integrity (266).

Pro-inflammatory cytokine processing and secretion

Besides pyroptosis and IEC extrusion, another key downstream effector function of inflammasomes is proteolytic processing and maturation of pro-IL-1β and pro-IL-18 and the release of the mature cytokines as well as other pro-inflammatory mediators (226). IECs only express and secrete IL-18 upon inflammasome activation, as expression of pro-IL-1β requires transcriptional priming (229, 230, 240, 267). The mature cytokines are secreted via GSDMD pores and play a key role in recruitment of immune cells such as natural killer cells and neutrophils involved in coordination and amplification of the immune response and eventually bacterial clearance (267).

The NAIP/NLRC4 inflammasome

Ligands and mechanism of activation

NLRC4 was initially identified to play a role for detection of intracellular *S*.Tm within macrophages (268). Importantly, the PAMPs that activate NLRC4 are key bacterial virulence factors, namely flagellin, the molecular building block of the flagellum (269, 270), and the T3SS (271). In contrast to other inflammasome pathways, NLRC4 does not directly function as a PRR, but upstream NAIP(s) bind bacterial PAMPs and subsequently recruit and activate NRLC4 (226). Specifically, a conserved region of flagellin is detected by murine NAIP5 and NAIP6 (272–274), T3SS rod proteins are recognized by NAIP2 (272, 274) and NAIP1 binds T3SS needle subunits (275, 276). Murine NAIPs recognize T3SS components from both *S*.Tm (rod protein PrgJ (271, 272, 274) and needle protein PrgI (276, 277)) and *S. flexneri* (rod protein MxiI (271, 275) and needle protein MxiH (276)), as well as other bacterial species. In contrast to mice, humans only encode a single NAIP that recognizes flagellin (278) as well as T3SS rod (279) and needle proteins (274, 276, 277) from *S*.Tm, *S. flexneri* and other bacterial species. Importantly, neither human nor murine NAIPs detect *S*.Tm T3SS-2 rod or needle proteins (271, 279), which highlights intracellular downregulation of T3SS-1 and expression of T3SS-2 as an important strategy for immune evasion. Upon ligand binding, NAIPs associate with NLRC4 and induce its oligomerization to form the inflammasome complex (272, 274, 280) (Figure 4).

Intestinal epithelial NAIP/NRLC4

As mentioned above, epithelial NAIP/NRLC4 plays a crucial role in the early defense against *S*.Tm infection. The IEC-intrinsic NAIP/NLRC4 inflammasome restricts intraepithelial bacterial loads *in vivo* and in murine enteroids by triggering prompt and specific extrusion of infected IECs (231, 234, 236, 281). By restricting intraepithelial colonization, IEC NAIP/NLRC4 furthermore limits breaching of the epithelial barrier and systemic spread (227, 236) as well as epithelial disintegration and destructive inflammation to maintain tissue integrity and functionality at later time points p.i. (231). *S*.Tm downregulates the expression of NAIP ligands during transcytosis within the SCV to evade inflammasome activation in innate immune cells of the lamina propria. On the other hand, epithelial NAIP/NLRC4 is perfectly positioned for pathogen detection, as flagella and the T3SS-1 are crucial virulence factors for IEC invasion (227). In particular, cytosolic *S*.Tm that are primed for re-invasion express high levels of NAIP ligands and promptly induce IEC extrusion to efficiently restrict this fast growing intraepithelial subpopulation (118, 130, 240, 281). Besides its role during *S*.Tm infection, epithelial NAIP/NLRC4 also fulfills a protective function during infection with *Citrobacter rodentium*, an extracellular adherent, attaching/effacing pathogen

of mice that models enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *E. coli* infection in humans (233).

While NAIP/NLRC4 constitutes the main inflammasome pathway in the murine intestinal epithelium (227, 236, 239, 281), it appears to play a less significant role in human IECs due to low expression levels of NAIP and NLRC4 (240, 242). Strikingly, murine NAIP/NLRC4 efficiently restricts also *S. flexneri* colonization of the intestinal epithelium, which begins to explain the natural resistance of mice to shigellosis, and highlights *Nlrc4*-deficient mice as a valuable *in vivo* model for *S. flexneri* infection (208).
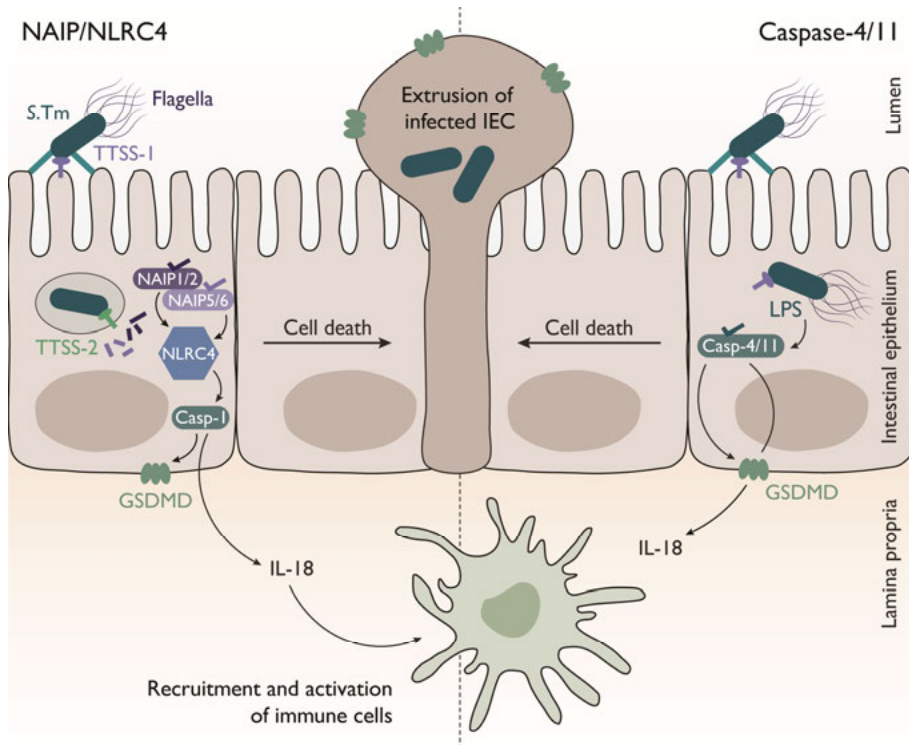

Figure 4. NAIP/NLRC4 and caspase-4/11 inflammasomes of the intestinal epithelium. Murine NAIP1/2 detect T3SS needle and rod components, respectively, and NAIP5/6 detect flagellin, while a single human NAIP detects all of these ligands in the cytosol. Upon ligand binding, NAIPs recruit NLRC4 and induce its activation for inflammasome formation and downstream processing of caspase-1 (Casp-1). Human caspase-4 and murine caspase-11 directly bind cytosolic LPS and act as both sensor and executor of the non-canonical inflammasome pathway. Inflammasome activation via either pathway ensures prompt extrusion of infected IECs to restrict epithelial colonization and preserve barrier integrity. Upon proteolytic processing, inflammatory caspases-1/4/11 cleave GSDMD to release an N-terminal cytotoxic domain that inserts into the membrane to form pores and induce a lytic mode of cell death termed pyroptosis. Caspases-1 and -4 furthermore process the pro-inflammatory cytokine IL-18 that is released via the GSDMD pores for recruitment and activation of immune cells.

The non-canonical caspase-4/11 inflammasome

Ligands and mechanism of activation

The term 'non-canonical inflammasome' was introduced with the discovery that caspase-11 was activated by non-canonical inflammasome ligands during bacterial infection of macrophages (247). In this non-canonical pathway, murine caspase-11 and human caspase-4 (and in some cases -5) function as both inflammasome sensor and executor. These caspases directly bind cytosolic LPS, which induces oligomerization and caspase autoprocessing for activation (246, 248, 250). Non-canonical inflammasomes induce pyroptosis by caspase-4/11-mediated cleavage of GSDMD (253). While activation of murine caspase-11 does not allow for direct cytokine maturation in IECs (247, 253, 282), human caspase-4 directly cleaves pro-IL-18 (and pro-IL-1β) for secretion of mature cytokines (232, 245, 249, 251, 282) (Figure 4).

Guanylate-binding proteins (GBPs) assist in non-canonical inflammasome activation as they directly bind LPS on the bacterial surface and expose its inflammasome-activating moiety, lipid A, for efficient caspase-4/11 recruitment and activation (249, 251, 283–288). Upon lysis of the BCV, GBP1 instantly relocates to the bacterial surface, where it binds LPS and recruits GBP2-4 to form a GBP coat (249, 251, 289, 290). This coat in turn recruits caspase-4 and promotes its LPS binding to initiate inflammasome assembly at the bacterial surface (249, 251). Furthermore, bacterial outer membrane vesicles have also been implied in cytosolic LPS delivery for activation of the caspase-11 inflammasome by cytosolic or extracellular bacterial pathogens (288, 291).

Intestinal epithelial non-canonical caspase-4/11 inflammasomes

S.Tm infection not only induces caspase-11-dependent pyroptosis in macrophages (244), but caspase-4/11-induced cell death and extrusion also reduce intraepithelial *S*.Tm loads to specifically restrict cytosolic bacterial replication (232, 250). Thereby, the non-canonical inflammasome plays a key role in the defense against *S*.Tm infection in the human intestinal epithelium in particular (240, 242). This species-specific relevance is again due to differential expression levels, as production of caspase-11 in murine IECs requires transcriptional priming, whereas caspase-4 expression is significant in human IECs at steady state (228, 239, 240). Intriguingly, *S. flexneri* efficiently evades non-canonical inflammasome-mediated restriction by blocking the signaling cascade at multiple levels via secreted T3SS effectors (241, 290, 292–294).

Inhibition of inflammasome-induced cell death by *Shigella*

The *S. flexneri* effector OspC3 inhibits caspase-4/11-mediated pyroptosis and IL-18 secretion to maintain the pathogen's cytosolic replicative niche (241, 293, 295). Mechanistically, ADP-riboxanation, a post-translational modification of

caspase-4/11, prevents activation via catalytic autoprocessing (293). Thereby, the ADP-riboxanase activity of OspC3 is stimulated by binding to calmodulin as a host cell cofactor (296). OspC3 may even modify already activated caspase-4/11 by ADP-riboxanation to inhibit proteolytic cleavage of downstream substrates, however, it preferably binds substrate-free caspases (293, 296). Two related enterobacterial extracellular pathogens, EPEC and *C. rodentium*, also secrete a T3SS effector, NleF, to inhibit the proteolytic activity of caspase-4/11 in IECs (297). This further highlights the importance of the non-canonical inflammasome in restricting enterobacterial colonization of the intestinal epithelium. The role of *S. flexneri* OspC3 during intestinal epithelial colonization is further discussed in **Paper III**.

Upstream of caspase-4/11, the *S. flexneri* T3SS effector IpaH9.8 ubiquitinates GBP1 and other GBPs to target them for proteasomal degradation, reduce LPS exposure and promote *S. flexneri* intracellular replication (289, 290, 292). Thereby, IpaH9.8 cooperates with OpsC3 to inhibit caspase-4/11 activation, pyroptotic cell death and IEC extrusion (251, 298). Furthermore, GBP coating of the bacterial surface inhibits unipolar localization of IcsA and actin-tail formation for cell-to-cell spread, which is also counteracted by IpaH9.8 (284, 289, 290). To further restrict caspase-4/11 activation, *S. flexneri* intracellularly modifies the composition of its LPS to produce a hypoacylated variant with reduced inflammasome-stimulatory capacity (299). Similarly, human-restricted *Salmonella* Paratyphi evades the non-canonical inflammasome via the production of long LPS O-antigen moieties that shield the innermost lipid A part from recognition by caspase-4 (300).

The *S. flexneri* T3SS effector IpaH7.8 is another ubiquitin ligase of the IpaH family and targets GSDMD for proteasomal degradation (294). As IpaH7.8 binds and modifies human but not mouse GSDMD, this effector may contribute to the species-specificity for *S. flexneri* infection (294, 301–303). In murine macrophages, IpaH7.8 also ubiquitinates the inflammasome sensor NLRP1B to target it for proteasomal degradation. Instead of inhibiting inflammasome activation, this functional degradation releases the NLRP1B C-terminal domain for inflammasome formation and caspase-1 activation (304). Importantly, human NLRP1 is not degraded by IpaH7.8, which might further contribute to species specificity.

IpgD, another *S. flexneri* effector, blocks ATP release from infected cells, which is an important DAMP that activates the NLRP3 inflammasome (305). Rearrangements of the plasma membrane concomitant with bacterial invasion induce ATP secretion via the membrane mechanosensor PIEZO1 (306). In addition, periplasmic *S. flexneri* apyrase degrades extracellular ATP to further reduce NLRP3 inflammasome activation (307). While NLRP3 is not expressed in the intestinal epithelium (228, 230, 245, 282), restriction of ATP release from IECs may prevent inflammasome activation in recruited innate immune cells at later time points of infection. Additional *S. flexneri* effectors are involved in the inhibition of other modes of cell death (202, 265, 308), but

to what extent they contribute to *S. flexneri* colonization of the intestinal epithelium remains unclear.

Experimental infection models and the emergence of enteroids and colonoids

Commonly used cell lines and animal models

Cell line models for enterobacterial infection

Traditionally, enterobacterial infections of the epithelium have been studied in tumor-derived or immortalized epithelial cell lines, often with verification of phenotypes in animal models. These studies have generated a valuable resource of detailed molecular mechanisms for individual bacterial effectors and their interaction with host cell targets, as cell lines are relatively easy to manipulate genetically and their low complexity allows for high spatial and temporal resolution (102, 309). Commonly used epithelial cell lines comprise e.g. the cervical adenocarcinoma cell line HeLa, transformed canine kidney (MDCK) cells as well as the intestinal epithelial colon carcinoma cell lines Caco-2, HT-29 and T84 (102). Caco-2 and HT-29 cells can be differentiated into polarized epithelial cells with microvilli when grown in a permeable transwell culture system (102), and the HT-29-MTX mucus-secreting subclone enables to study the role of mucus during bacterial adhesion and invasion (310). In addition, macrophage cell lines and murine bone marrow-derived macrophages have been used to characterize innate immune responses such as inflammasome cascades (102).

Animal models for *S.*Tm and *S. flexneri* infection

Laboratory mice are the most commonly used animal model, but in contrast to gastroenteric manifestations in humans, *S.*Tm induces typhoid-like, systemic disease in mice (30). Therefore, bovine infection models were initially used to mimic the pathogenesis in humans, as cattle are natural hosts for *S.*Tm (30, 311). In addition, bovine, porcine and rabbit ligated ileal loops have proven a valuable model for *S.*Tm enterocolitis (311, 312). The finding that *S.*Tm induces enterocolitis in streptomycin-pretreated mice has revolutionized the field as the system is amenable to genetic manipulations on both the pathogen and host cell side (32, 313). In this model, antibiotic treatment transiently disrupts colonization resistance by the microbiota to allow for *S.*Tm intestinal colonization.

For *S. flexneri* infection, a variety of animal models have been explored, but due to the pathogen's primate-restricted nature, none of them exhibits all aspects of human shigellosis (314). While Rhesus macaques recapitulate the pathogenesis seen in humans, this model has been abandoned for ethical reasons (314). The traditional gold standard *in vivo* assay for virulence is the so-

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called Sereny test, a guinea pig keratojunctivitis model (315). In addition, rabbit and guinea pig ileal loops (316), intrarectal inoculation in guinea pigs (317), infection of infant rabbits (210) as well as other guinea pig and rabbit infection models have been established (314). The extraintestinal mouse intranasal pneumonia model (318, 319) and zebrafish (320) also support *S. flexneri* infection, but with limited applicability for the study of bacteria-IEC interactions. Only recently, the discovery that the NAIP/NLRC4 inflammasome restricts colonization of the murine intestinal epithelium by *S. flexneri* has established *Nlrc4*-deficient mice as a valuable, genetically amenable *in vivo* model that recapitulates key symptoms of human shigellosis (208).

Limitations of traditionally used model systems

Animal models are complex and hence temporal resolution and mechanistic studies are limited. In contrast, cell line models offer higher resolution and mechanistic insight, but with limited physiological relevance, and translation of results from cell lines to animal models or human patients is far from trivial. Importantly, epithelial cell line models differ significantly from the primary intestinal epithelium *in vivo*, as they generally lack primary cell architecture and display reduced cell surface glycosylation and an altered glycocalyx composition compared to the intestine *in vivo* (321). Despite responsiveness of cell lines such as Caco-2 to certain inflammasome ligands, transformed and tumor-derived cell lines generally display reduced expression of inflammasome constituents (228, 240) and appear hypersusceptible to enterobacterial infection (84, 240, 322). Traditionally, inflammasome responses have been studied in macrophage cell lines or bone marrow-derived macrophages. Upon contact with lamina propria immune cells *in vivo*, however, bacteria have often down-regulated their expression of inflammasome ligands (227).

Intestinal organoid models

Establishment of intestinal epithelial organoids

Recently, the development of organotypic cultures has revolutionized the research of enteropathogenic infections and many other fields. Intestinal organoids contain non-transformed IECs and provide a unique opportunity to bridge the gap between the traditionally used cell line and *in vivo* models. Providing an optimized tradeoff between experimental simplicity and primary cell features, they allow to study physiologically relevant processes at high spatial and temporal resolution. Intestinal organoids can be established from induced or embryonic pluripotent stem cells (323), (induced) fetal intestinal progenitors (324, 325) or adult ISCs (326–329). Pluripotent stem cell-derived intestinal organoids are large and cystic in their appearance, and contain epithelium-surrounding mesenchymal cells (323). Organoids established from

crypt-residing, adult ISCs are referred to as enteroids or colonoids, depending on their segment of origin, and comprise a single layer of polarized IECs surrounding a central lumen. Enteroids and colonoids are purely epithelial and, in contrast to pluripotent stem cell-derived organoids that require long-term culture or transplantation for full differentiation (330), they recapitulate the 3D architecture of the intestinal epithelium with crypts extending from the central lumen after relatively short culture periods (326–328) (Figure 5).
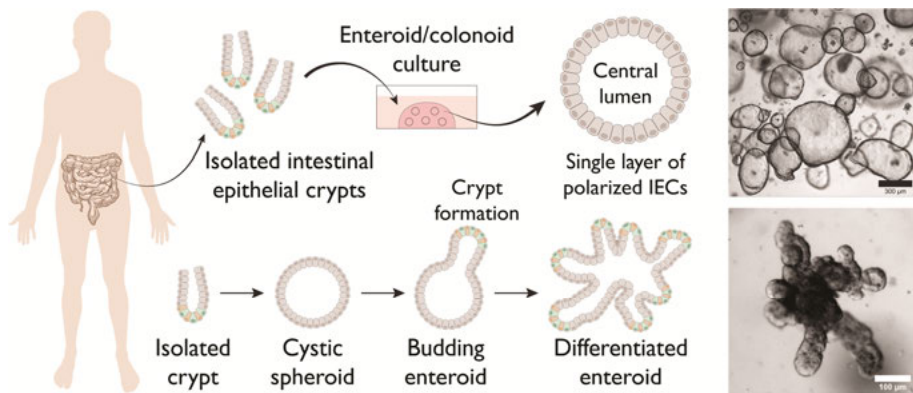
Figure 5. Enteroid/colonoid establishment and culture. Enteroids and colonoids are intestinal organoids established from isolated crypt-residing adult ISCs. (Top) When embedded an ECM-mimicking gel and provided with the necessary growth factors, isolated crypts form cystic spheroids comprising a single layer of polarized IECs that surrounds the central lumen. The image shows a culture of human enteroids in Wnt-containing expansion medium with enteroids largely consisting of proliferative cells. Scale bar: 300 μm. (Bottom) When cultured in the absence of the stem cell-maintaining growth factor Wnt, spontaneous symmetry breaking events lead to the formation of crypts extending from the lumen in budding enteroids, until eventually differentiated enteroids with various IEC types are obtained. The image depicts a differentiated murine enteroid. Scale bar: 100 μm.

Enteroid and colonoid culture

Once established, enteroids and colonoids can be passaged and maintained in culture for several months, and compatibility with cryopreservation allows for long-term storage (327–329). To maintain the stem cell niche and capacity for continuous self-renewal, enteroids and colonoids are cultured in extracellular matrix-mimicking gels and provided with a complex cocktail of growth factors. Culture media contain agonists of Wnt and epidermal growth factor (EGF) signaling involved in stem cell maintenance, as well as inhibitors of the bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) differentiation-inducing pathways (331). Murine enteroids cultured under these conditions comprise all IEC types present in the intestine *in vivo* and self-organize into crypts with ISCs and Paneth cells at their bottom and villus domains facing the central lumen (327). Wnt production by Paneth cells in murine enteroids is sufficient for maintenance of the stem cell niche (7). On

the other hand, culture of murine colonoids and human enteroids or colonoids requires exogenous supplementation with Wnt in the culture medium ('expansion medium'). Uniform distribution of this growth factor results in cystic structures with high proliferation potential termed 'spheroids' that almost exclusively consist of low-differentiation ISCs and transit amplifying cells (326, 328, 329). Upon withdrawal of Wnt ('differentiation medium'), these spheroids lose their proliferative capacity and adopt a more differentiated morphology with crypt and villus domains containing different absorptive and secretory IEC types (326, 328). Recently, improved culture conditions for human enteroids have been developed to maintain the stem cell niche while generating all differentiated IEC types. In particular, addition of IL-22 to the culture medium has been found to induce the formation of Paneth cells (332). Furthermore, addition of RANKL (receptor activator of NF-κB ligand) and TNF (tumor necrosis factor) to the culture medium induces the formation of M cells (333–335).

Modelling enteropathogen infection in enteroids and colonoids

Since their discovery, a variety of intestinal organoid-based models has been employed for the study of infections by viral (e.g. 237, 336–344), eukaryotic (e.g. 345–354) and bacterial enteropathogens (Table 2). These models have allowed to overcome host range restriction and lack of relevant *in vitro* culture models that support the entire lifecycle for many of these pathogens (171, 173, 337, 340, 343, 346, 350, 353, 354) and are expected to reduce the need for animal experiments. Studies of bacterial infection in human and murine enteroids and colonoids are summarized in Table 2. In addition enteroids and colonoids from other species (344, 349, 353, 355–357), pluripotent stem cell-derived intestinal organoids (338, 358–365) and fetal progenitor-derived intestinal organoids (336, 366, 367) have also been employed for the study of enteropathogenic infections.

Enteroid- and colonoid-based experimental models for enteropathogen infection

The use of 3D enteroids and colonoids for enteropathogenic infections is complicated by the fact that the apical surface of the epithelium, from where invasion naturally emanates, faces the enclosed lumen of the organoid and is not readily accessible for infection. Experimentally, this limitation has been overcome in several ways, giving rise to different classes of enteroid/colonoid-based models (Table 2). (i) In the simplest experimental model, enteroids/colonoids are infected in suspension from the basal side (231, 345, 368). Prior fragmentation of the enteroids/colonoids may thereby expose the apical face of the epithelium for invasion (339, 341, 343, 352, 355, 365, 369, 370), but with limited physiological relevance. (ii) Further, enteropathogens can be microinjected into the central lumen (350, 352, 371–375; **Papers I, II**) to enable
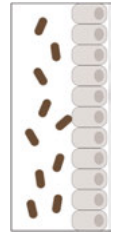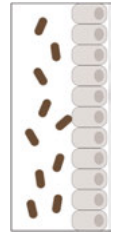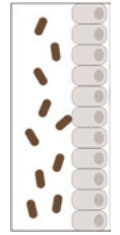
epithelial invasion via the apical side from an enclosed luminal environment, which more recapitulates the infection route *in vivo*. To circumvent the technical challenge of luminal microinjection and increase throughput, (iii) inverted polarity 'apical-out' enteroids/colonoids have been established to allow for apical invasion in bulk suspension culture. (356, 376). (iv) Finally, to improve compatibility with (live cell) imaging by providing a stable imaging plane, enteroids and colonoids can be dissociated into single cells and seeded as 2D monolayers on coated transwell filters (377–380) or flexible hydrogels (381, 382; **Paper I**). Similar to 3D enteroids and colonoids, these monolayers can be differentiated by complete removal or reduced concentration of Wnt in the culture medium (377, 378, 380, 382).

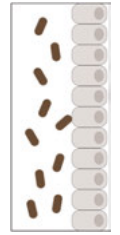**Physiological relevance of enteroid and colonoid models**

Enteroid- and colonoid-derived monolayers display transmembrane mucin expression and glycosylation patterns that recapitulate the intestinal surface *in vivo*, and glycosylation increases further upon differentiation (85). Such an *in vivo*-relevant epithelial glycocalyx is key for studies of apical surface targeting, adhesion and invasion by enteropathogens. Furthermore, enteroids and colonoids express inflammasome components at higher levels than commonly used cell lines and comparable to primary IECs *in vivo* (228, 240), which is crucial for assessing the role of epithelial inflammasomes during enteropathogen infection (208, 231, 237, 239, 240, 251, 261, 281). Enteroid/colonoid-derived monolayers treated with RANKL and TNF have been applied to study the role of M cells during bacterial infection (85, 173, 369, 383). In addition, co-cultures with different types of immune cells (384) have been explored to study how immune cells affect pathogen-IEC interactions (186, 361, 363, 385).

To further improve the physiological relevance of enteroid/colonoid models, the native intestinal crypt-villus geometry has been mimicked by seeding IECs on 3D scaffolds. Chemical gradients of growth factors across these scaffolds induce *in vivo*-like cellular distribution with a replicative stem cell niche in the crypts and progressive upward migration of IECs as they differentiate (382, 398). Enteroid and colonoid-based perfused organ-on-chip models include flow conditions to mimic physiological shear stress (389, 399–401), peristaltic-like movements and co-culture with endothelial cells (400) or macrophages (399). These organ-on-chip models have been applied to study infection by pathogenic *E. coli* (389, 397). Finally, the combination of microengineered 3D scaffolds and microfluidics has yielded intestine chips with highly physiological tissue architecture, growth factor gradients and flow conditions (402, 403). This model has proven applicable to study infection with the intestinal parasite *Cryptosporidium parvum* and supports completion of its complex lifecycle (403).

Table 2. Overview of bacterial infection studies in human and murine enteroids and colonoids

	3D bulk infection	3D microinjection	3D apical-out polarity	2D monolayers
Human enteroids		*S.*Tm (**Papers II, III**), *S. flexneri* (**Paper III**)	*Listeria monocytogenes* (376), *S.*Tm (376)	*Escherichia coli*: AIEC (386), EAEC (27, 185, 380, 387–389), EHEC (185), EPEC (185, 380, 385), ETEC (385, 390–392), ExPEC (393); *Listeria monocytogenes* (394); *S. flexneri* (149, 171, 173, 185, 186, 188, 251; **Paper III**); *S.*Tm (85, 185, 240, 322, 334, 395; **Papers I, III**); *Salmonella* Typhi (185); *Yersinia pseudotuberculosis* (383)
Human colonoids	*Staphylococcus aureus* (365)	*S.*Tm (**Paper III**), *S. flexneri* (**Paper III**)		*Escherichia coli*: EAEC (27, 185, 380, 387, 388), EHEC (185, 377, 396, 397), EPEC (185, 380); *S. flexneri* (146, 171, 173, 185; **Paper III**); *S.*Tm (85, 185; **Paper III**); *Salmonella* Typhi (185)
Murine enteroids	*Listeria monocytogenes* (370), *Mycobacterium avium* (369), *S.*Tm (34, 231, 368, 369)	*Lawsonia intracellularis* (372), *Listeria monocytogenes* (371), *S.*Tm (374, 375; **Paper I, II**)		*S.*Tm (231, 239, 240, 261; **Paper I**), *S. flexneri* (**Paper III**)
Murine colonoids		*Yersinia pseudotuberculosis* (373)		

AIEC, adherent invasive *E. coli*; EAEC, enteraggregative *E. coli*; EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*

Present investigations

Overall aim

Enterobacterial infections caused by *Salmonella* and *Shigella* are a leading cause of foodborne gastroenteritis globally, with considerable burden on healthcare and agricultural systems (13). The commonly used prototype strains *S*.Tm and *S. flexneri* colonize the intestinal lumen and face soluble and surface-bound mucus layers separating them from the underlying IEC surface (203, 404). A variety of mechanisms have been proposed for how this protective layer is overcome by either pathogen to invade IECs and establish an intraepithelial niche (203, 405). Within host cells, *S*.Tm and *S. flexneri* are exposed to innate immune receptors such as epithelium-intrinsic inflammasomes, cytosolic multiprotein complexes assembling upon PAMP detection to restrict epithelial pathogen loads by inducing cell death and extrusion of infected cells along with proinflammatory cytokine secretion (267). Hence, interactions with the apical IEC surface and epithelial inflammasomes are crucial determinants for successful colonization of the host and the pathogenesis of *S*.Tm and *S. flexneri.*

The mechanisms of *S*.Tm and *S. flexneri* host cell targeting and the role of inflammasomes have been studied extensively in transformed or immortalized cell lines or animal models. However, commonly used cell lines lack important aspects of primary tissue architecture with a drastically reduced cell surface glycocalyx of altered composition and low expression of inflammasome components compared to the intestine *in vivo* (228, 240). On the other hand, temporal resolution and experimental controllability are limited in high-complexity animal models. The more recent development of enteroids and colonoids, intestinal organoids established from adult stem cells of the small intestine or colon, respectively, offer a non-transformed and purely epithelial model (326–329) with the potential for infection studies with relevant host cell physiology at high temporal and spatial resolution. However, these models have been insufficiently explored for dynamic analyses of *S*.Tm and *S. flexneri* infections. In particular, their potential for a high temporal resolution map of the various interconnected stages and bacteria-host cell interactions underlying epithelial colonization remains to be exploited.

The overall aims of this thesis were:

1. To establish enteroid and colonoid 3D and 2D models compatible with live cell time-lapse imaging and quantitative image analysis to study *S*.Tm and *S. flexneri* luminal and epithelial colonization and host responses to infection in real-time (addressed in **Papers I-III**)

2. To model the dynamics of *S*.Tm and *S. flexneri* enteroid/colonoid colonization and pinpoint the underlying virulence factor modules and their interaction with epithelial inflammasomes (addressed in **Papers I-III**)

3. To determine bacterial strategies and host cell surface characteristics governing *S*.Tm and *S. flexneri* targeting of the host cell surface to enable invasion (addressed in **Papers III-IV**)

Paper I: NAIP/NLRC4 inflammasome activation by *S*.Tm triggers prompt epithelial contractions

Background

The NAIP/NLRC4 inflammasome detects intracellular bacteria based on components of the bacterial T3SS and flagellin, the molecular building block of flagella (406). Inflammasome assembly induced upon binding these PAMPs activates inflammatory caspase-1 for downstream cleavage of GSDMD to form membrane pores and induce lytic cell death and secretion of pro-inflammatory cytokines via these pores (226). Murine IECs abundantly express components of the NAIP/NRLC4 signaling cascade (227, 236, 240) and this inflammasome has been shown to induce prompt and specific extrusion of *S*.Tm-infected IECs (234, 236). Thereby, the NAIP/NLRC4-caspase-1 axis plays a major role in limiting intraepithelial *S*.Tm loads *in vivo* (234, 236) and in murine enteroids (231, 239, 240). Furthermore, restriction of epithelial *S*.Tm colonization is crucial for maintenance of barrier integrity to restrict systemic dissemination (227, 236) and prevent exacerbated inflammation (231).

However, uncontrolled excessive IEC extrusion may reduce IEC numbers to a level that is insufficient to uphold epithelial integrity and may jeopardize barrier function. How the intestinal epithelium navigates this delicate balance of infected IEC extrusion and barrier maintenance at a tissue-scale level remains to be elucidated. Epithelial layer compaction in infected regions would be one intriguing possibility. Collective epithelial cell movement has been observed during *Listeria monocytogenes* actin-based lateral spread (394). Thereby, collective inward movement of bystander cells toward an invasion focus resulted in mound formation and collective expulsion of multiple

infected cells to restrict *L. monocytogenes* intraepithelial expansion. However, most of the experiments were conducted in transformed MDCK cells, and acute cell movements during the initial phase of invasion, or consequences of this mounding response for maintenance of barrier integrity were not assessed.

Summary

In **Paper I**, we identified a novel role for epithelial NAIP/NLRC4 in eliciting tissue-scale contractions upon *S*.Tm invasion of murine enteroid-derived 2D monolayers. Time-lapse imaging combined with an optical flow analysis platform (407) allowed studying the dynamicity of enteroid-derived 2D monolayers in response the detection of *S*.Tm T3SS-1 and flagellin. Focal contractions were triggered within the first minutes p.i. and propagated to the surrounding cells, resulting in a collective inward movement that involved several hundred IECs. Such contraction epicenters were completely absent from NAIP/NLRC4-deficient monolayers or upon infection of wildtype monolayers with non-invasive bacteria lacking the T3SS-1. Experiments in genetically chimeric monolayers with both NAIP/NLRC4-proficient and -deficient parts revealed that inflammasome activation is required for initiation, but not for propagation of the contractile response. Epithelial contractions were dependent on caspase-1 and GDSMD downstream of *S*.Tm-induced inflammasome activation. Furthermore, the high temporal resolution of the enteroid-based model enabled disentangling focal epithelial contractions on one hand, and IEC death and extrusion, on the other hand, as two separable consequences of NAIP/NLRC4 signaling, revealing that contractions occur prior to IEC extrusion and cell death. Pharmacological inhibitors were employed to determine a dependence on ion fluxes and myosin contraction. We propose a model in which ion fluxes resulting from sublytic GSDMD pore formation induce myosin contraction pulses to propagate the contractile response. Induction of such tissue-scale contractions serves to preserve epithelial integrity through increased local cell packaging at sites of *S*.Tm invasion and, eventually, infected IEC extrusion (Figure 6).

My role in this project was the analysis of tissue-scale contractions in murine 3D enteroids by quantifying the reduction in enteroid cross-sectional area following *S*.Tm microinjection. In line with the data from 2D monolayers, an epithelial contractile response was observed only upon injection of *S*.Tm wt, but not in mock-injected enteroids or enteroids injected with the T3SS-1-deficient Δ*invG* mutant. In addition, my work on establishing a protocol for the generation of chimeric enteroid-derived monolayers (231) was an important foundation for this study and I contributed to the statistical analysis of the data.
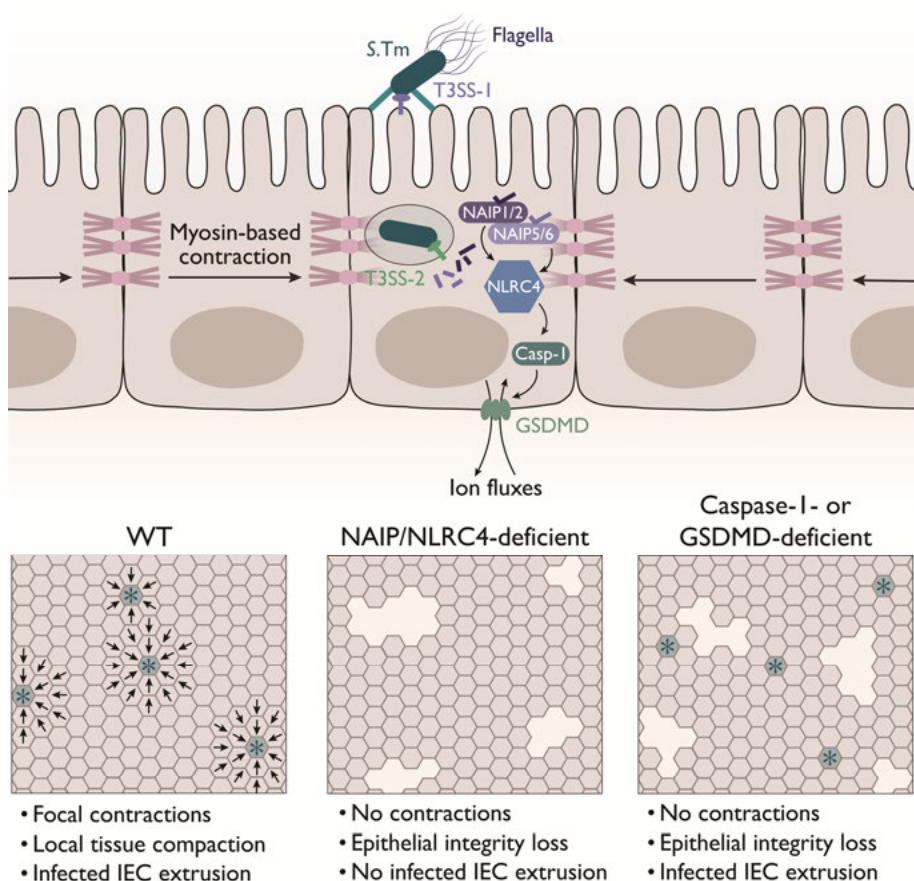
Figure 6. Graphical summary of Paper I. *S*.Tm infection of murine enteroid-derived monolayers causes focal contractions by activation of the NAIP/NLRC4 inflammasome. Ion fluxes via sublytic GSDMD pores induce myosin-based propagation of the contractile response, which results in local tissue compaction for maintenance of barrier integrity upon subsequent extrusion of the infected IEC. While contractions are strictly dependent on NAIP/NLRC4, caspase-1 and GSDMD, inflammasome activation and IEC extrusion (*) still occur in the absence of caspase-1 or GSDMD.

Paper II: *S*.Tm epithelial colonization cycle in human and murine enteroids

Background

Various organoid-based systems have been used to study enterobacterial infections. However, human 3D enteroid microinjection models based on luminal delivery of bacteria to allow for invasion from the inward-facing apical side had prior to this study only been applied for the study of parasitic infection by *C. parvum* (350). Importantly, human enteroids supported completion

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of the various stages of the pathogen's complex epithelial life cycle. Microinjections of murine enteroids (374, 375) and induced pluripotent stem cell-derived intestinal organoids (358–360, 362, 363) have proven a valuable tool to model *S*.Tm infection. In contrast to 2D models, in which dissociated enteroid/colonoid-derived IECs are seeded as a flat cell layer, the enclosed lumen of 3D enteroids offers a unique opportunity to study luminal colonization. Previous studies have implicated that bacteria-containing extruded IECs might contribute to luminal re-seeding (118, 130). However, due to the high complexity and limited resolution of *in vivo* models, it has remained challenging to assess potential collaboration and cross-feeding between luminal and epithelial *S*.Tm populations.
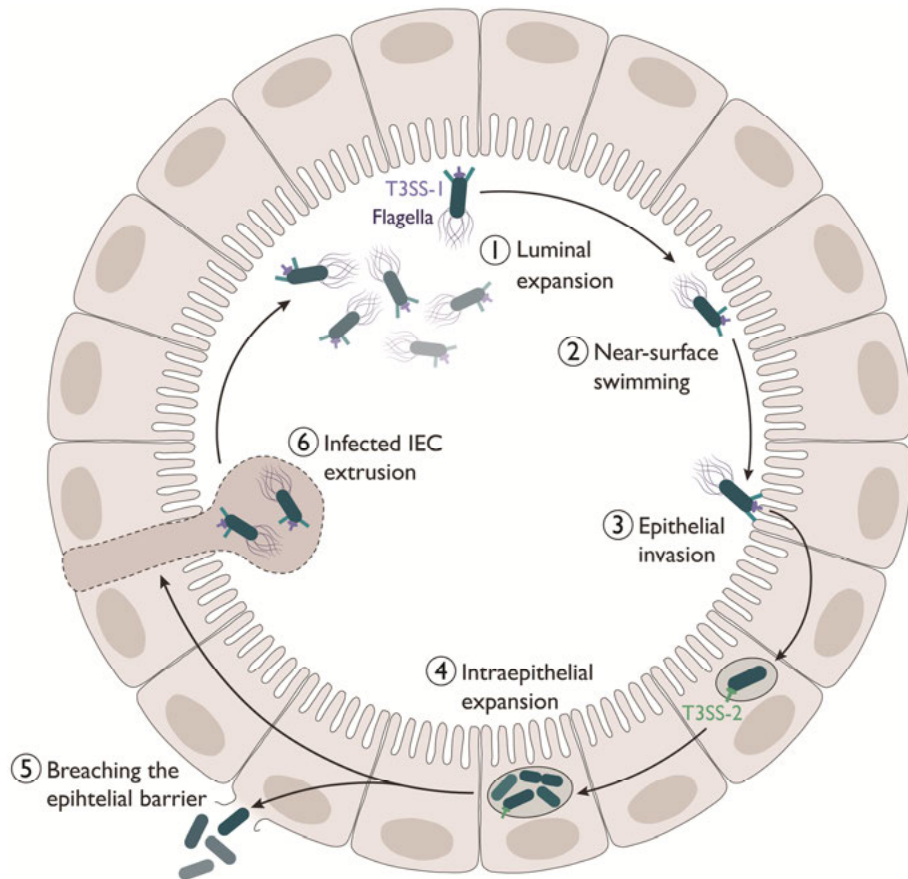
Figure 7. Graphical summary of Paper II. Human and murine enteroid microinjection supports all steps of the *S*.Tm epithelial colonization cycle: ① luminal expansion, ② near-surface swimming along the epithelial surface, ③ IEC invasion, ④ intraepithelial replication, resulting in either ⑤ epithelial barrier breaching, or ⑥ infected IEC extrusion. Luminal planktonic growth ① and cycling through IECs (via steps ③, ④ and ⑥) represent two parallel mechanisms for efficient luminal colonization.

Summary

In **Paper II**, we established a microinjection model for *S*.Tm infection of human and murine enteroids and applied it to address the contribution of major *S*.Tm virulence factor systems to luminal colonization. To recapitulate the *S*.Tm epithelial colonization cycle in non-transformed IECs, we followed microinjected bacteria by time-lapse microscopy to track the individual stages in real-time. During the first hours p.i., we observed luminal expansion based on the increase in fluorescence from a constitutive bacterial reporter. *S*.Tm remained motile upon luminal microinjection and reached the epithelial surface within seconds to engage in near-surface swimming. Using a SPI-2-dependent intracellular vacuolar reporter (p*ssaG*-GFP), we furthermore identified epithelial invasion foci that increased in size over time, indicative of intracellular replication. The signal from a cytosolic intracellular reporter (p*uhpT*-GFP), on the other hand, was barely detectable in our model system, which is in agreement with a previous study employing this reporter in enteroids (240). While a short-lived cytosolic population might still be present, massive hyperreplication is likely prevented by prompt inflammasome activation in primary IECs (240, 281). Supporting this notion, we also observed frequent extrusion of bacteria-harboring IECs in the enteroid microinjection model and this phenomenon was even more pronounced in murine than human enteroids. Finally, breaching of the epithelial barrier and bacterial release on the basolateral side was noted at later time points and could be interpreted as a proxy for systemic spread.

When assessing *S*.Tm virulence factors involved in luminal colonization, we found that flagellar motility was dispensable, as gravitational sedimentation allowed non-motile strains to efficiently reach the epithelial surface at the enteroid bottom plane for invasion in this simplified model devoid of secreted mucus. Non-invasive strains lacking the T3SS-1 ($\Delta invG$), or its four main effectors SipA, SopB, SopE and SopE2 ($\Delta 4$), on the other hand, displayed reduced expansion within the enteroid lumen. Epithelial invasion might boost luminal colonization by (i) altering the growth conditions in the luminal environment or (ii) luminal reseeding via intraepithelial replication and infected IEC extrusion. To distinguish between these alternative mechanisms experimentally, we took advantage of a mixed consortium infection approach (408). Co-infections with genetically barcoded invasive (wt) and T3SS-1-deficient *S*.Tm ($\Delta invG$) allowed us to refute the first scenario, as the non-invasive mutant was similarly impaired in luminal colonization during co-infections. Hence, we concluded that *S*.Tm exploits cycling through IECs to efficiently colonize the enteroid lumen (Figure 7), a finding that was later confirmed by an elegant experimental approach *in vivo* in mice (129). By engineering an *S*.Tm strain that self-destructs upon reaching the host cell cytosol, the authors of that study showed that cytosolic escape and subsequent IEC extrusion contribute significantly to intestinal colonization.

Paper III: Divergent *S*.Tm and *S. flexneri* epithelial colonization strategies in human enteroids and colonoids

Background

Despite close relatedness and overlapping sets of virulence factors, *S*.Tm and *S. flexneri* display a few key differences in their pathogenesis. *S*.Tm infects a broad range of host species and employs flagellar motility and a variety of adhesins to target the epithelial surface for invasion. *S. flexneri*, on the other hand, is primate-restricted and non-motile, and the role of adhesins for epithelial colonization is controversial, as alternative mechanisms have been suggested too. Within non-transformed IECs, *S*.Tm is largely restricted to a vacuolar intracellular compartment, whereas *S. flexneri* adopts a cytosolic lifestyle. Epithelial inflammasome-induced cell death and extrusion of infected IECs are a key innate immune barrier towards bacterial colonization of the intestinal epithelium. Hence, it is not surprising that enteropathogens have developed countermeasures, often in the form of inhibitory effectors, to restrict inflammasome activation. In the case of *S. flexneri*, a set of effectors comprising OspC3 (241, 293, 295), IpaH7.8 (294) and IpaH9.8 (289, 290, 292) have been described to suppress inflammasome cascades at different levels.

While studies in immortalized and tumor-derived cell lines have provided a plethora of molecular mechanisms for individual virulence factors and bacterial effectors, we lack a synthesis of how virulence effector modules and their dynamic interaction with host cell responses shape *S*.Tm and *S. flexneri* colonization strategies in non-transformed IECs.

Summary

In **Paper III**, we employed time-lapse imaging of 3D and 2D human enteroids and colonoids infected with *S*.Tm and *S. flexneri* in parallel to identify virulence factor modules dictating their divergent epithelial colonization strategies. Enteroid and colonoid luminal microinjections established that while both wt and non-flagellated *S*.Tm readily invade the epithelium, *S. flexneri* invasion from the apical face required epithelial damage. To assess the role of adhesins during apical targeting, we designed two novel adhesion assays compatible with the enteroid/colonoid microinjection model and found that non-flagellated *S*.Tm and *S. flexneri* differ markedly in their adhesion potential. Combined removal of flagella and the giant adhesin SiiE encoded on SPI-4 (52) effectively transformed *S*.Tm into a *S. flexneri*-like bacterium floating atop the epithelial surface, and unable to adhere stably and invade. Hence, we concluded that adhesins play a key role in the transition from Brownian-like floating to stable binding and T3SS-mediated docking to the apical epithelial

surface. In addition, the momentum generated by flagellar motility also promoted some degree of stable adhesion and invasion in the absence of SPI-4.
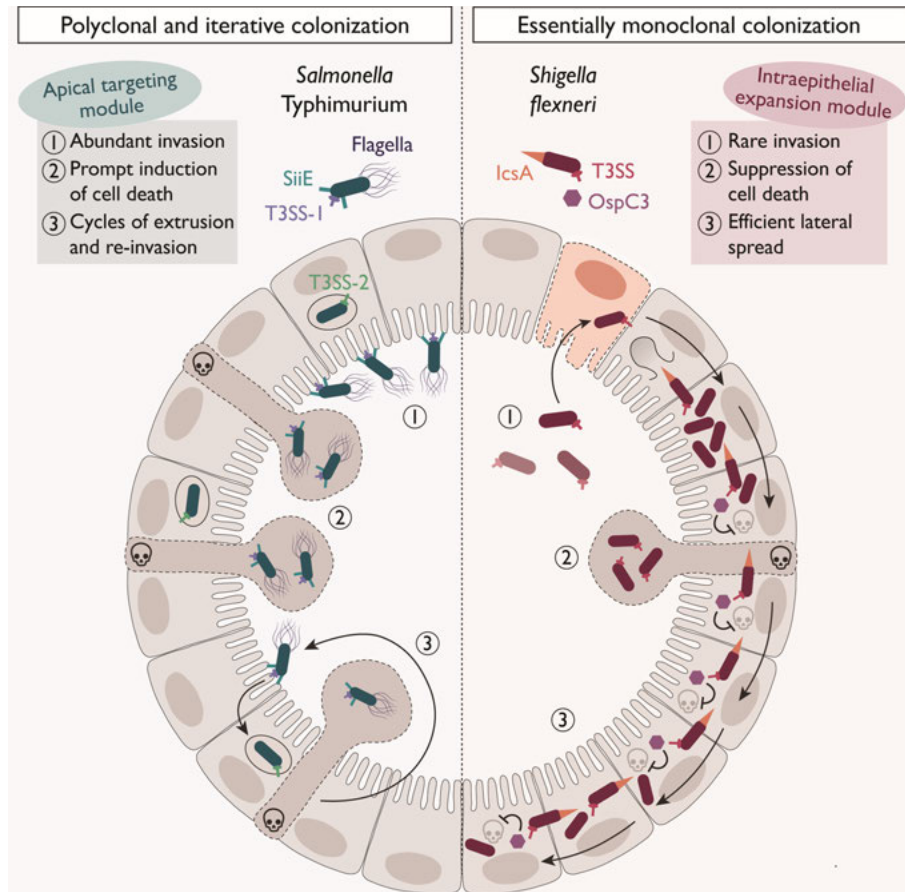

Figure 8. Graphical summary of Paper III. *S.*Tm and *S. flexneri* employ alternative strategies for successful colonization of the intestinal epithelium. Equipped with an apical targeting module combining flagellar motility and the SPI-4-encoded SiiE adhesin system, *S.*Tm efficiently scans the epithelial surface and encounters a high density of permissive binding sites. This results in abundant invasion from the lumen, but also prompt induction of host cell death and extrusion of infected IECs for luminal reseeding, giving rise to a polyclonal and iterative colonization strategy. Due to the lack of flagella and adhesins, *S. flexneri* invasion from the apical face is exceptionally rare and requires pre-existing epithelial damage. Suppression of inflammatory caspase-mediated cell death via OspC3 allows *S. flexneri* to persist within the epithelium and expand efficiently via IcsA-mediated cell-to-cell spread. Concerted action of these two effectors yields an intraepithelial expansion module at the core of this essentially monoclonal colonization strategy. (Modified from Paper III, Figure 6).

Next, we sought to assess the fate of intraepithelial *S.*Tm and *S. flexneri* populations following successful invasion. Switching to a 2D monolayer model

and inclusion of a centrifugation step to force contact and synchronize invasion by non-motile bacteria enabled sparse *S. flexneri* invasion foci. Employing our recently developed protocol to assess cell death in enteroid/colonoid-derived monolayers (409) revealed a striking capacity of *S. flexneri* to spread intraepithelially while efficiently restricting IEC death. This remarkable intraepithelial expansion was dependent on OspC3-mediated delay of caspase-induced IEC death to allow for IcsA-driven lateral spread to neighboring IECs. In conclusion, we described a *S.*Tm apical targeting module that integrates flagellar motility and the SPI-4-encoded adhesin system with T3SS-1 to accomplish a high density of adhesion and invasion events. *S.*Tm invasion foci, however, are short-lived and promptly terminated by IEC death and extrusion, resulting in a polyclonal iterative epithelial colonization strategy. Lacking such an apical targeting module, *S. flexneri* relies on external factors such as preexisting damage for rare apical invasion of the epithelium, which it compensates for by an intraepithelial expansion module. Therein, coupling of OspC3-mediated delay of inflammasome-induced host cell death with IcsA-driven lateral spread allows *S. flexneri* to escape the IEC death response, resulting in an essentially monoclonal colonization strategy (Figure 8).

Paper IV: A naked host cell membrane is the optimal surface for *S.*Tm targeting

Background

Cell line models have been extensively used for mechanistic studies of bacterial invasion processes and have established comprehensive maps of underlying virulence factors. However, systematic studies of host cell features involved in cell surface targeting have not been accomplished to the same extent. Secreted and transmembrane mucins, as well as other glycostructures, are important constituents of the intestinal epithelial cell surface to keep commensals at bay and restrict invasion by enterobacterial pathogens (10). Not surprisingly, cell surface glycans play a key role in the interaction of entero-pathogenic bacteria such as *S.*Tm with their host cells and have been described to function either as a barrier (21, 22, 55, 85, 410–415) or as ligands for bacterial adhesins (24–27, 50, 51, 54, 55, 63, 391, 416). In addition, membrane composition, and cholesterol levels in particular (66, 417–420), also affect *S.*Tm host cell targeting, as the T3SS-1 and flagella can directly bind to the membrane (64–66). Disentangling the contribution of these various host cell surface features to efficient *S.*Tm targeting *in vivo* or in enteroid/colonoid models with a high-complexity glycocalyx is challenging. Mechanistic studies of isolated host cell surface determinants would be facilitated by a simplistic model for bottom-up reconstruction of surface features compatible with high-throughput screening.

Summary

In **Paper IV**, we explored suspension-growing leukemia K562 cells as a simplistic, high-throughput and genetically amenable model for *S*.Tm infection. We established early interaction and invasion assays in 96-well format based on automated, flow cytometry-based readout of constitutive (p*rpsM*-mCherry) or intracellular vacuolar, SPI-2-inducible (p*ssaG*-GFP) bacterial fluorescent reporters (Figure 9). Comparison with a well-established infection model confirmed that invasion is mediated by the same set of T3SS-1 effectors required for invasion of HeLa cells, with an additional contribution of SipA, that has been shown to play a major role for the invasion of non-transformed human and murine IECs in enteroids/colonoids and *in vivo* (84, 85). Several studies have reported preferential *S*.Tm invasion of mitotic cells (43, 419, 421), but have proposed either increased cholesterol levels (419) or rounding up of adherent cells during mitosis (43) as the underlying mechanism. The K562 model seemed ideally suited to disentangle these features, as morphology is constant throughout the cell cycle in suspension-growing cells. Our results confirmed increased cholesterol levels in mitotic cells, which however did not result in preferential mitotic cell targeting in our model. Rather, cells with high cholesterol levels displayed increased bacterial association throughout the cell cycle.

Global transcriptome and proteome analysis revealed low levels of glycosaminoglycan and transmembrane mucin expression and hinted towards a minimal glycocalyx in K562. To functionally assess the role of cell surface glycans as a barrier or adhesin ligands, we employed enzymatic trimming of the glycocalyx. Neuraminidase treatment to cleave off terminal sialic acid moieties, but not N-glycosidase F treatment targeting N-glycans, promoted *S*.Tm-host cell interaction, which suggests a barrier function of the endogenous K562 glycocalyx (Figure 6). Furthermore, we found that ectopically expressed GP2 and MUC1 formed a size-dependent steric barrier towards *S*.Tm targeting, despite their documented role as ligands for *S*.Tm adhesins (24, 26). Parallel analysis of K562 and previously published differentiated human enteroid-derived monolayer IEC transcriptomes and proteomes, along with glycan sequence predictions and lectin stainings suggested IEC-relevant, although less diverse mucin-type O-glycosylation in K562. Finally, we found that, analogous to host cell surface glycoconjugates, even bacterial cell surface appendages such the giant adhesin SiiE hampered bacterial association with the low complexity cell surface in this simplistic model (Figure 6). This is in stark contrast with the crucial role of SiiE in non-transformed human IECs (see **Paper III**). Therefore, we conclude that the plethora of bacterial adhesins is an inevitable consequence of the fact that pathogens are faced with a dense glycocalyx during intestinal colonization. However, a naked host cell membrane would be the 'dream scenario' for *S*.Tm T3SS-1-mediated targeting from the pathogen's point of view.
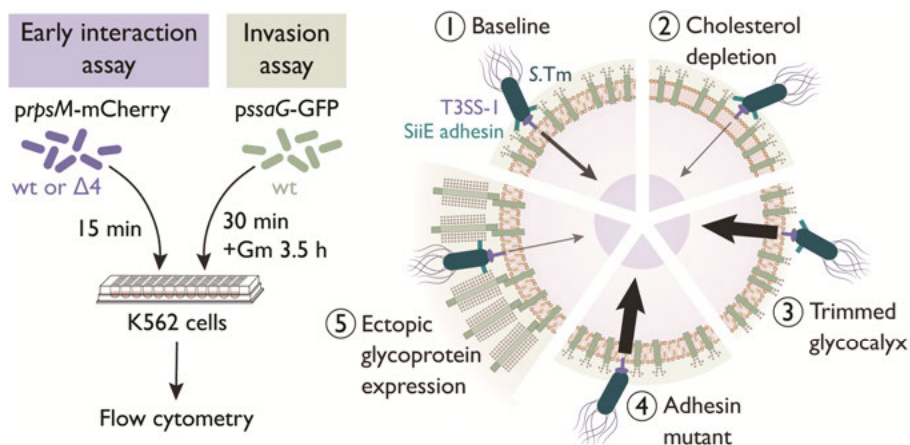
Figure 9. Graphical summary of Paper IV. (Left) A simplistic high-throughput *S*.Tm infection model compatible with automated flow cytometry was developed in suspension-growing K562 cells. The early interaction assay is based on a 15 min co-incubation of K562 cells with *S*.Tm wt (adhesion and early invasion) or Δ4 (adhesion only) harboring the constitutive reporter p*rpsM*-mCherry. The invasion assay employs 30 min infection followed by 3.5 h gentamicin (Gm) treatment for maturation of an intracellular vacuolar p*ssaG*-GFP reporter. (Modified from Paper IV, Figure 1). (Right) Various host cell and bacterial surface manipulations indicate ① appreciable invasion efficiency at baseline, ② which is reduced upon depletion of cell surface cholesterol, ③ increased by cell surface glycocalyx trimming or ④ removal of bacterial surface structures such as the SiiE adhesin, and ⑤ a barrier effect of ectopic glycoprotein expression, despite their function as adhesin ligands. This suggests a naked host cell membrane rich in cholesterol as the optimal surface for *S*.Tm targeting.

Conclusion and future perspectives

In this thesis work, we established murine and human enteroid and colonoid 3D microinjection and 2D IEC monolayer models for infection with the prototype enterobacterial pathogens *S*.Tm and *S. flexneri*, which are compatible with live cell imaging and quantitative image analysis (Papers I-III). In contrast to traditionally employed transformed or immortalized cell line models, these enteroids/colonoids retain primary tissue characteristics such as cell surface composition and *in vivo*-like expression levels of inflammasome cascade components. Furthermore, they allow for mechanistic studies at increased spatial and temporal resolution compared to animal models. As a high-throughput complement, we also developed a simplistic and genetically amenable model based on suspension-growing K562 cells (Paper IV). Bacterial and host cell mechanisms that promote or restrict interaction with the apical surface of the epithelium, as well as interaction with IEC inflammasomes are crucial determinants for successful establishment of infection. Hence, the models developed herein were employed to study (i) luminal and epithelial colonization in real-time (Papers II, III), (ii) virulence factor modules that shape the epithelial colonization strategies of *S*.Tm and *S. flexneri* (Paper III), (iii) their interactions with epithelial inflammasomes (Papers I, III), and (iv) bacterial strategies for targeting the IEC apical surface for invasion (Paper III, IV).

In **Paper I**, we found that *S*.Tm invasion induces tissue-scale contractions in 2D enteroid-derived monolayers and microinjected 3D enteroids via activation of the IEC NAIP/NLRC4 inflammasome, calcium fluxes through GSDMD sublytic pores, and myosin-based propagation of the contractile response to surrounding IECs. This response preceded extrusion of infected IECs and resulted in local tissue compaction to prevent loss of epithelial integrity during infection. Thereby, this study describes an important and previously unnoticed downstream consequence of IEC inflammasome activation that protects the host from *S*.Tm invasion-induced tissue damage. Whether this protective response also occurs upon infection with other enteropathogens or following activation of other inflammasomes remains an intriguing topic for future research.

In **Paper II**, we described that luminal microinjection of human and murine enteroids supports all stages of the *S*.Tm epithelial infection cycle, namely luminal expansion, epithelial invasion, intraepithelial replication, basolateral release and breaching of the epithelial barrier as well as infected IEC

extrusion. Furthermore, we identified cycles of epithelial invasion, intraepithelial replication and reemergence via infected IEC extrusion as an additional pathway operating in parallel with luminal expansion to ensure efficient colonization of both luminal and epithelial niches. This highlights enteroids and colonoids as a valuable tool for dynamic and *in vivo*-relevant live-cell analyses at high temporal and spatial resolution, and will spark many future studies of interconnected stages and collaborating bacterial subpopulations during enterobacterial colonization of the intestinal epithelium.

In **Paper III**, we conducted parallel infections with *S*.Tm and *S. flexneri* in 3D and 2D human enteroid and colonoid models to determine virulence factor modules that shape their divergent epithelial colonization strategies. We found that *S*.Tm is equipped with an apical targeting module combining flagellar motility and the SPI-4-encoded SiiE adhesin system to establish abundant, although short-lived, invasion events, and foster a polyclonal and iterative epithelial colonization strategy. *S. flexneri*, on the other hand, relies on external factors to establish rare epithelial invasion foci. An intraepithelial expansion module compensates for this low invasion efficiency by coupling OspC3-mediated delay of caspase-dependent IEC death with IcsA-driven lateral spread. Investigating the contribution of additional *S. flexneri* effectors that suppress the inflammasome cascades upstream (IpaH9.8 targeting GBP-mediated LPS release for non-canonical inflammasome activation) and downstream (IpaH7.8 inhibiting GSDMD) will be an interesting topic for future investigations.

In **Paper IV**, we employed our simplistic K562 infection model coupled with automated flow cytometry analysis to characterize host cell surface determinants involved in *S*.Tm targeting. While membrane cholesterol promoted *S*.Tm invasion, the endogenous K562 glycocalyx, as well as ectopically expressed glycoproteins with adhesin ligand function, represented a barrier towards *S*.Tm targeting. Analogous to host cell surface glycostructures, also bacterial appendages such as the giant adhesin SiiE hindered interaction with the low-complexity K562 cell surface. This led us to conclude that while adhesins are necessary to overcome the glycocalyx barrier that covers the apical face of the intestinal epithelium, a naked cell membrane rich in cholesterol represents the optimal surface for *S*.Tm T3SS-1-mediated targeting. We anticipate that live-cell imaging using microfluidic devices will allow to map preferential targeting of this preferred host cell surface in real-time. Furthermore, validation of the size-dependent glycocalyx barrier effect via expression of truncated transmembrane mucin versions with reduced numbers of VNTRs in K562 cells to modulate its thickness will be an intriguing future addition to this study.

In conclusion, the methodological advancements and concepts described herein will provide a valuable base for future mechanistic insights into additional intricacies of enteropathogen-host cell interactions at high spatial and temporal resolution.

Popular science summary

Our intestines are inhabited by billions of microbes. While these commensal microbes are not harmful, and in certain cases even beneficial for human health, uptake of contaminated food or water may introduce disease-causing pathogens into the digestive tract. In the gut, these pathogenic microbes disrupt the well-functioning interplay between the human host and its commensal microbes. Commensal microbes generally do not spread beyond the digestive tract and throughout the body, as our intestines are protected by a firewall, the intestinal epithelium, that separates the microbial community in the lumen from underlying tissues and the bloodstream. Pathogens, on the other hand, attack this firewall and try to invade intestinal epithelial cells to hide from immune cells and other antimicrobial defense mechanisms inside the epithelium. This is however not completely successful, and the presence of pathogenic microbes in the gut lumen and epithelium triggers a variety of immune processes to counteract the onslaught and maintain the epithelial firewall. These processes result in common disease symptoms such as diarrhea, stomach ache or vomiting.

The pathogenic bacteria *Salmonella* and *Shigella* are common causes of foodborne diarrhea and important model organisms in the laboratory to understand how such bacteria trigger disease. The epithelium that lines the gut is the first point of contact between these bacteria and the human body, and fulfills an important firewall function. Therefore, it is particularly interesting to study the interaction of pathogenic bacteria with intestinal epithelial host cells. One attractive model system for that purpose is the culture of 'mini-guts', also called enteroids and colonoids, small balloon-like structures formed by intestinal epithelial cells of the small or large intestine, respectively. These structures can be established from human patient biopsies obtained during routine surgery and may be cultured in the laboratory for several months or even years. Enteroids and colonoids have several advantages over other model systems. Firstly, they offer a valuable alternative to animal experiments and allow for infection with pathogens such as *Shigella* that normally only infect humans, apes and monkeys. Secondly, enteroids and colonoids are structurally and functionally more similar to the human gut than commonly used laboratory cell lines that have often been derived from cancer tissue. To mimic foodborne infection, pathogenic microbes can be microinjected into the enteroid

or colonoid lumen, the inside of the balloon, or the epithelium can be cultured as a two-dimensional flat layer and infected from the top.

In this thesis work, we studied the mechanisms of *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* (hereafter referred to as *Salmonella* and *Shigella*) infection in enteroids and colonoids. Specifically, we were interested in the strategies these bacteria use to reach and invade the intestinal epithelium, as well as countermeasures by epithelial cells to uphold the firewall. Two important aspects of the epithelial firewall are carbohydrate structures attached to the surface of epithelial cells, the so-called glycocalyx, and inflammasomes. Inflammasomes are an early alert system for pathogenic intruders inside epithelial cells. Once they detect a molecular clue that points towards an invading pathogen, they set off the alarm and activate a chain reaction that eventually results in cell death and expulsion of the infected cell from the epithelial layer.

In **Paper I** of this thesis, we found that the chain reaction triggered by the inflammasome alert system also induces local contractions of the epithelium at sites of *Salmonella* invasion. The infected cell uses myosin to pull on actin strings, and transmit the contraction to neighboring epithelial cells. This all happens before the infected cell died and prepares its neighbors to quickly fill the gap left behind as it is expelled. When we blocked the inflammasome alert system in our experiments, *Salmonella* infection did not trigger any contractions. Instead, holes formed in the epithelial layer and the firewall was overcome by the bacteria. This highlights inflammasome-induced epithelial contractions as a new important feature of the intestinal firewall.

In **Paper II**, we microinjected *Salmonella* into the enteroid lumen, the inside of the balloon, and used microscopy to follow the infection in real-time. We could see how *Salmonella* can both grow in the lumen and invade epithelial cells. The bacteria could also grow inside the epithelium and occasionally managed to escape to the outside of the enteroid balloon. Most often, however, infected epithelial cells were expelled into the lumen fast enough to prevent this. To our surprise, we found that *Salmonella* in expelled cells actually contributed to the high numbers of bacteria accumulating in the lumen as they were released from dead cell carcasses.

In **Paper III**, we compared the toolboxes, consisting of virulence factors, and strategies that *Salmonella* and *Shigella* use to colonize the epithelium. We mapped how *Salmonella* combines a rope-like swimming motor, the flagella, and sticky extensions, termed adhesins, to efficiently reach and bind to the epithelial surface. This resulted in parallel infection of many cells, but these cells were also quickly expelled from the epithelium. *Shigella*, on the other hand, does have neither flagella nor adhesins and appears remarkably poor at invading epithelial cells from the lumen-facing apical side. Therefore, it chooses a completely different strategy and other tools. One tool shuts off the inflammasome alert system, while the other allows *Shigella* to spread from one epithelial cell to the next and massively expand its numbers within the

epithelium. That way, both *Salmonella* and *Shigella* successfully intrude the epithelium, but use completely different tools and strategies to do so.

In **Paper IV**, we used a simpler model that allowed us to manipulate the host cell surface in different ways and identify factors that strengthen or weaken the barrier towards invading *Salmonella*. We found that carbohydrate structures attached to the cell surface, even the ones that *Salmonella* adhesins like to stick to, strengthen the glycocalyx barrier. While our experiments from the previous study showed that adhesins are required to stick to the epithelial surface of the intestine and allow bacteria to invade epithelial cells, we here conclude that *Salmonella* would actually prefer a naked host cell membrane for invasion.

Altogether, this thesis provides novel models and techniques to study interactions of *Salmonella*, *Shigella*, and potentially also other pathogenic microbes, with their host cells. We also expanded the knowledge about the firewall function of the intestinal epithelium during *Salmonella* and *Shigella* infection, relying on the elaborate glycocalyx barrier at the cell surface and the inflammasome early alert system.

Deutsche Zusammenfassung

Unser Darm beherbergt natürlicherweise Milliarden von Mikroben, die sogenannte Mikrobiota. Während diese Mikroben keinesfalls schädlich, und in manchen Fällen sogar nützlich für die menschliche Gesundheit sind, kann die Aufnahme von kontaminierter Nahrung oder Wasser den Darm Krankheitserregern, sogenannten Pathogenen, aussetzen. Diese krankheitserregenden Mikroben zerstören das Gleichgewicht zwischen Wirt (dem Menschen) und der Mikrobiota und greifen unser Darmepithel an. Das Darmepithel gleicht einer Schutzmauer, die tieferliegende Gewebe und den Blutkreislauf von Mikroben im Inneren des Darmes, dem sogenannten Lumen, trennt. Dies ist eine sehr effektive Barriere gegenüber der Mikrobiota sowie vielen Pathogenen. Allerdings können manche Krankheitserreger in Darmepithelzellen eindringen, um sich dort zu vermehren und vor dem Immunsystem und anderen antimikrobiellen Prozessen zu verstecken. Dies gelingt jedoch nur teilweise und die Gegenwart dieser Krankheitserreger löst verschiedene Immunantworten aus, um den Angriff auf die epitheliale Schutz-mauer abzuwehren. Diese Reaktionen des Körpers auf die Infektion äussern sich dann in den üblichen Krankheitssymptomen wie Durchfall, Bauchschmerzen oder Erbrechen.

Bakterielle Krankheitserreger wie Salmonellen oder Shigellen sind eine häufige Ursache von Lebensmittelvergiftungen und somit wichtige Modellorganismen für die Untersuchung dieser Krankheiten im Labor. Da das Darmepithel der erste Kontaktpunkt zwischen Krankheitserregern und dem menschlichen Körper darstellt, ist es besonders interessant, die Interaktion des Epithels mit Pathogenen zu studieren. Ein attraktives Modellsystem zu diesem Zweck sind Darmorganoide, kleine, ballonartige Gebilde aus Darmepithelzellen des Dünn- oder Dickdarmes. Darmorganoide können mithilfe von menschlichen Biopsien aus der Chirurgie etabliert, und für mehrere Monate oder sogar Jahre im Labor kultiviert werden. Gegenüber anderen Modellsystemen haben Darmorganoide mehrere Vorteile. Zum einen bieten sie eine wertvolle Alternative zu Tierexperimenten und ermöglichen sogar Infektionen mit Krankheitserregern wie Shigellen, die normalerweise nur Menschen und Affen infizieren. Zum anderen ähneln sie strukturell und funktionell viel stärker dem wirklichen Darmepithel in Mensch oder Tier, als häufig verwendete Zelllinien, die in den meisten Fällen aus Tumorgewebe hergestellt wurden. Um eine natürliche Infektion nachzuahmen, können Krankheitserreger in das Innere von

Darmorganoiden mikroinjiziert werden, oder das Darmepithel kann als zweidimensionaler Zellteppich kultiviert und von oben infiziert werden.

In meiner Doktorarbeit wurden Infektionsmechanismen von Salmonellen und Shigellen (*Salmonella enterica* serovar Typhimurium und *Shigella flexneri*, um genau zu sein) in Darmorganoiden untersucht. Wir waren intressiert daran, welche Strategien diese Bakterien verwenden, um das Darmepithel zu erreichen und in Epithelzellen einzudringen. Ausserdem wollten wir verstehen, wie das menschliche Darmepithel seine Funktion als Schutzmauer während einer Infektion aufrechterhalten kann. Zwei wichtige Aspekte dieser Schutzmauer sind Kohlenhydratstrukturen, die an der Oberfläche des Darmepithels angebracht sind, die sogenannte Glykokalyx, und Inflammasome. Inflammasome sind Frühwarnsysteme für krankheitserregende Eindringlinge im Innern des Darmepithels. Wenn ein Hinweis auf einen Krankheitserreger gefunden wird, schlägt das Inflammasom Alarm und löst eine Kettenreaktion aus, die den kontrollierten Zelltod der infizierten Epithelzelle und ihren Ausstoss aus dem Darmepithel zur Folge hat.

In der ersten Publikation dieser Doktorarbeit konnten wir sehen, dass die durch Inflammasome verursachte Kettenreaktion lokale Kontraktionen des Darmepithels an denjenigen Stellen hervorrief, wo Salmonellen in Epithelzellen eingedrungen waren. Die infizierte Zelle benutzte dann Myosin, um an Aktinseilen zu ziehen und die Kontraktion an benachbarte Zellen weiterzuleiten. All das passierte vor dem Tod der infizierten Zelle und bereitete Nachbarzellen darauf vor, die enstehende Lücke schnell zu schliessen, sobald die infizierte Zelle aus dem Darmepithel ausgestossen wurde. Sobald wir das Inflammasom-Alarmsystem in unseren Experimenten blockierten, konnten wir keine Kontraktionen mehr beobachten. Stattdessen bildeten sich Löcher im Darmepithel und die Salmonellen konnten die Schutzmauer durchbrechen. Dies zeigte uns inflammasomabhängige Kontraktionen als eine neue wichtige Reaktion des Körpers zur Aufrechterhaltung der epithelialen Schutz-mauer auf.

In der zweiten Publikation mikroinjizierten wir Salmonellen in das Innere von Darmorganoiden und folgten der Infektion mithilfe von Mikroskopie in Realzeit. Wir konnten sehen, wie Salmonellen im Inneren von Darmorganoiden wachsen und erfolgreich in Darmepithelzellen eindrangen. Die Bakterien wuchsen auch innerhalb des Darmepithels und konnten in manchen Fällen sogar aus dem Darmorganoid-Ballon ausbrechen. Meist wurde dies jedoch durch die schnelle Ausstossung der infizierten Zellen in das Lumen verhindert. Zu unserer Überraschung entdeckten wir, dass Salmonellen aus diesen ausgestossenen Epithelzellüberresten zu den hohen Bakterienzahlen im Innern der Darmorganoide beitrugen.

Im dritten Artikel verglichen wir die von Salmonellen und Shigellen verwendeten Werkzeugkasten, bestehend aus Virulenzfaktoren, und Strategien zur Besiedlung des Darmepithels. Wir entdeckten dabei, dass Salmonellen seilartige Schwimmmotoren, die Fagellen, und klebrige Oberflächenstrukturen, sogenannte Adhesine, benötigen, um effizient ins Darmepithel eindringen zu

können. Dies resultierte darin, dass viele Bakterien parallel ins Epithel eindrangen. Allerdings wurden diese eingedrungenen Salmonellen auch rasch wieder aus dem Epithel ausgestossen. Shigellen haben weder Flagellen noch Adhesine und konnten daher nur sehr selten von der dem Lumen zugewandten Seite ins Darmepithel eindringen. Deshalb verwendeten sie eine komplett andere Strategie zur Besiedlung des Darmepithels, bei der ein Werkzeug das Inflammasom-Alarmsystem auschaltete, während ein anderes die schnelle Ausbreitung von Zelle zu Zelle und massive Shigellenvermehrung ermöglichte. Dies hat uns gezeigt, dass Salmonellen und Shigellen komplett unterschieldiche Werkzeuge und gegensätzliche Strategien zur erfolgreichen Besiedlung des Darmepithels verwenden.

Im vierten Artikel entwickelten wir ein einfacheres Modellsystem, das uns erlaubte, die Zelloberfläche auf verschiedene Arten zu verändern. Dabei fanden wir heraus, dass Kohlenhydratstrukturen auf der Zelloberfläche, und sogar solche, an denen Salmonellenadhesine gerne kleben bleiben, die Barrierefunktion der Glykokalyx verstärkten. Während unsere Experimente im vorherigen Artikel gezeigt hatten, dass Adhesine notwendig sind, damit Bakterien sich an der Oberfläche des Darmepithels festkleben können, entdeckten wir hier, dass eine nackte Zellmembran eigentlich die bevorzugte Oberfläche zur Eindringung von Salmonellen darstellen würde.

In ihrer Gesamtheit stellt diese Doktorarbeit neuartige Modellsysteme und Methoden zur Verfügung, um Interaktionen des Darmepithels mit Salmonellen und Shigellen, sowie potentenziell auch weiteren Krankheitserregern, zu studieren. Wir konnten ausserdem unser Wissen über die Schutzmauerfunktion des Darmepithels während Salmonellen- und Shigelleninfektionen erweitern, indem wir das Inflammasom-Alarmsystem und die Glykokalyxbarriere an der Epithelzelloberfläche untersuchten.

Acknowledgements

There are so many wonderful people I would like to thank at this point for making my PhD studies and the past six years in Uppsala the amazing experience they have been. Thank you all for your support, encouragement and friendship, and for simply being part of this chapter in my life.

First of all, a huge thank you to my supervisor **Mikael**. I could write a long list of all the things I'm thankful for, but let's keep it simple: I couldn't imagine a better supervisor than you. Thanks for giving me this opportunity to do my PhD in your group, for all the things I've learned from you, and for always keeping on encouraging me. Great thanks also to **Maria Letizia**, my co-supervisor. I'm very impressed and encouraged by the dedicated and determined researcher you are. Thanks for introducing me to the world of *Shigella*, my new favorite pathogen, and teaching me the art of graphical abstracts. Thanks also to my other co-supervisor, **Jenny**. Even though my project never ended up going the way it was initially planned, thanks for believing in the immunology in my projects. Another word of thanks goes to my examiner, **Anna-Karin**, thanks for your interest in my research throughout the past six years.

Another big round of applause to my two extremely talented master students keeping the K562 project alive when I was busy infecting organoids. **Willem**, thanks or sticking around in the midst of the pandemic to optimize K562 transfections. I'm still impressed by your independence in the lab and all you achieved during your time with us. **Johanna**, the artist, for the wonderful title image, and of course for all your countless experiments, the K562 manuscript would not have been part of this thesis without you.

Huge thanks also to my PhD siblings. **Viktor**, you were the big brother I never had, always encouraging and up for a chat or a coffee when I needed it. Thanks for teaching me all about tracking bacteria. **Chris**, my fellow flow cytometry wizard, without you and your funny anecdotes (of course provided without any context), my PhD life would have been a lot more boring. **Alexandra**, thanks for bringing this urgently needed extra sunshine into my life and spreading your impressive never-ending joy all around you. Thanks for believing in my Swedish and teaching me all about high-throughput monolayer invasion assays. **Finja**, my youngest PhD sister, thanks for never giving up on inviting me for fika or lunch, even when I once again was in another world with my noise-cancelling headphones on. Keep the glycobiology alive!

Thanks also to my fellow lab members, **Jens**, ruling over the microscopes (and a beautiful little island in Åland). What a privilege to always have Frankenscope performing at its best, my projects would only have been completed half-way without you. **Ana**, keeper of organoids and fellow scientist mum, thanks for making our lab such a well-oiled machinery. **Nora**, even though you've only been here for a year, it feels like it has been forever. Thanks for your friendship and for keeping us all safe from toxic chemicals. Thanks also to our satellite lab members, **Anjeela**, extending our work to the realm of *Giardia*, and **Julia**, the horse organoid lady.

The next round of thanks goes to the Sellin lab alumni, **Marie**, thanks for letting me take over your lab bench and for patiently speaking Swedish with me from the very beginning. **Pilar**, always up for scientific and outdoor adventures. I'm very impressed by who you are and super proud to be the second author of your paper. **Eva**, the *Shigella* queen, I'm still missing you in our office and around the lab. Thank you for the warm and friendly atmosphere you spread all around you. **Jorik**, never getting tired or answering my questions about organoids or R programming, thanks for introducing me to tidyverse. Thanks also to our former master students, **Nathalie**, **Isabel**, **Angelica**, **Margot** and **Thibault**, for providing this direly needed fresh energy boost to the group.

Many thanks also to all the past and current IPhA (IMBIM PhD association) board members who made the PhD journey so much more enjoyable. Thanks to **Aida**, for introducing me to IPhA, and to **Jenny**, **Elin** and **Tifaine**, my fellow IPhA mums. **Isa**, **Filip** and **Janina** thanks for the many evenings we just spent time together and had fun playing Pandemic or Dobble. A special thanks to **Xinran**, you are truly inspiring me in so many ways, thanks for going before me along the path to PhD defense and answering all the questions I had along the way. Thanks to the current IPhA board members, **Philip**, **Raphaela**, **Anna**, **Anusha** and **Erik** for keeping IPhA alive.

A great thank you also to the amazing administrative team at the IMBIM department, especially to **Veronica**, **Rehné** and **Malin**, I don't think any other department could be better organized. Thanks to **Eva** and **Anette** for keeping the course labs organized and making teaching the fun experience it was, and to all fellow PhD students teaching together with me. Thanks to **Susan**, **Hanna** and **Petra** taking good care of the MACS flow cytometer at ICM, and for always being available to answer all my questions. The K562 project could not have prospered without your support.

A huge thank you of course goes to my wonderful family, my amazing husband **Benjamin**, for always supporting me throughout the past years and listening to my complaints about experiments or instruments that didn't work, even though you probably had no clue what I was talking about. Thanks for complementing me in so many ways and being such a wonderful and patient father to our children, I love you! Huge thanks also to my little sun shines **Joan** and **Joëlle**, always welcoming me with joyful screams and a hug when

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U.I.O.G.D.

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 2081

Editor: The Dean of the Faculty of Medicine

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