

Partners in Crime: Investigating the role of human tetraspanins CD81 and CD9 during *Salmonella* invasion

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List of Abbreviations

ANOVA	Analysis of Variance
APC	Allophycocyanin
CD	Cluster of Differentiation
CRISPR	clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
EGF	Epidermal Growth Factor
EPEC	Enteropathogenic <i>Escherichia coli</i>
FACS	Fluorescence Activated Cell Sorting
GFP	Green fluorescent protein
GTP	Guanosine Triphosphate
HCV	Hepatitis C Virus
HCVcc	Hepatitis C virus cell culture
HCVpp	Hepatitis C virus pseudoparticle
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule 1 (CD54)
JAM-A	Junctional adhesion molecule A
LAMP1	Lysosomal-associated membrane protein 1
LEL	Large Extracellular Loop
mAbs	Monoclonal antibodies
MOI	Multiplicity of Infection
M β CD	Methyl- β -cyclodextrin
PLA	Proximity ligation Assay
Rck	Resistance to complement killing
SEL	Short Extracellular Loop
SCV	<i>Salmonella</i> containing vacuole
SPI	<i>Salmonella</i> Pathogenicity Island
S. Tm	<i>Salmonella</i> Typhimurium
T3SS-1	Type 3 secretion system 1
TEM	Tetraspanin-enriched microdomain
TNF- α	Tumor necrosis factor alpha

Abstract

Human CD81 and CD9 are members of the tetraspanin family of proteins which are characterized by a canonical structure of four transmembrane domains and two extracellular loop domains which protrude about 3-5 nm into the extracellular milieu. Tetraspanins are known as molecular facilitators, which assemble and organize cell surface receptors and partner molecules forming clusters known as tetraspanin-enriched microdomains (TEMs). They have been implicated to play various biological roles including facilitating infection processes of microbial pathogens (*i.e.*, as adhesion platforms, receptor signaling platforms, etc.). Following this premise, we investigated the role of the human tetraspanins, CD81 and CD9 in the context of *Salmonella* invasion to epithelial host cells by creating overexpression and knockout infection models. Here, we demonstrated that overexpression of CD81 and CD9 in HepG2 cells enhanced the invasion of various representative typhoidal and non-typhoidal *Salmonella* strains. Conversely, deletion of CD81 by CRISPR/Cas9 in intestinal epithelial cells (Caco2-Bbe1 and HT29-MTX-E12) attenuated *S. Typhimurium* invasion. We showed that blocking human CD81 with recombinant HCV-derived glycoprotein E2, as well as CD81-specific antibodies, reduced *S. Tm* invasion in a dose-dependent manner. Furthermore, sequestering cell surface cholesterol with methyl- β -cyclodextrin prior to *Salmonella* infection, suggested a CD81-dependent effect during bacterial invasion. Finally, immunofluorescence microscopy and proximity ligation assay revealed that both human tetraspanins CD81 and CD9 are co-recruited to the entry site of *Salmonella* exclusively during invasion but not during *Salmonella* adhesion to the host cell surface. Overall, we conclude that the human tetraspanins CD81 and CD9 are molecular partners facilitating *Salmonella* invasion into epithelial host cells.

Introduction

Bacteria and eukaryotic hosts have established intimate relationships with each other, spanning millions of years of co-evolutionary behaviors with each other within which time, they have devised specific adaptations to overcome the offenses and defenses of each other [1]–[3].

A good example of such an intimate host-bacterial pathogen relationship is that exemplified by *Salmonella* infection. *Salmonella* is a Gram-negative, rod-shaped facultative intracellular pathogen that causes gastroenteritis or enteric fever and represents a public health risk worldwide [4], [5]. Following ingestion of contaminated food or water, the bacteria survive the gastric acidity of the stomach and reach the small intestine where it preferentially invades cells such as the M cells. After transcytosis into the epithelium, it can then invade into macrophages and dendritic cells. This can then facilitate the spread of *Salmonella* further into nearby organs such as the spleen and the liver, causing inflammation and diarrhea to the human host and, in severe cases becomes a systemic life-threatening or persistent infection [6], [7].

1. *Salmonella*

A. Taxonomy and importance as a foodborne pathogen

Salmonella is a gram-negative rod-shaped bacterium which continues to be a major cause of bacterial enteric illness throughout the world. Both the European Food Safety Authority (EFSA) and the Centers for Disease Control and Prevention have identified *Salmonella* sp. as the main culprit of food poisoning globally [8], [9].

The genus of *Salmonella* belongs to the family *Enterobacteriaceae* of which there are currently two taxonomically recognized species- *Salmonella bongori* and *Salmonella enterica*. *S. enterica* is further subdivided into six subspecies designated by a Roman Numeral (refer to Figure 1).

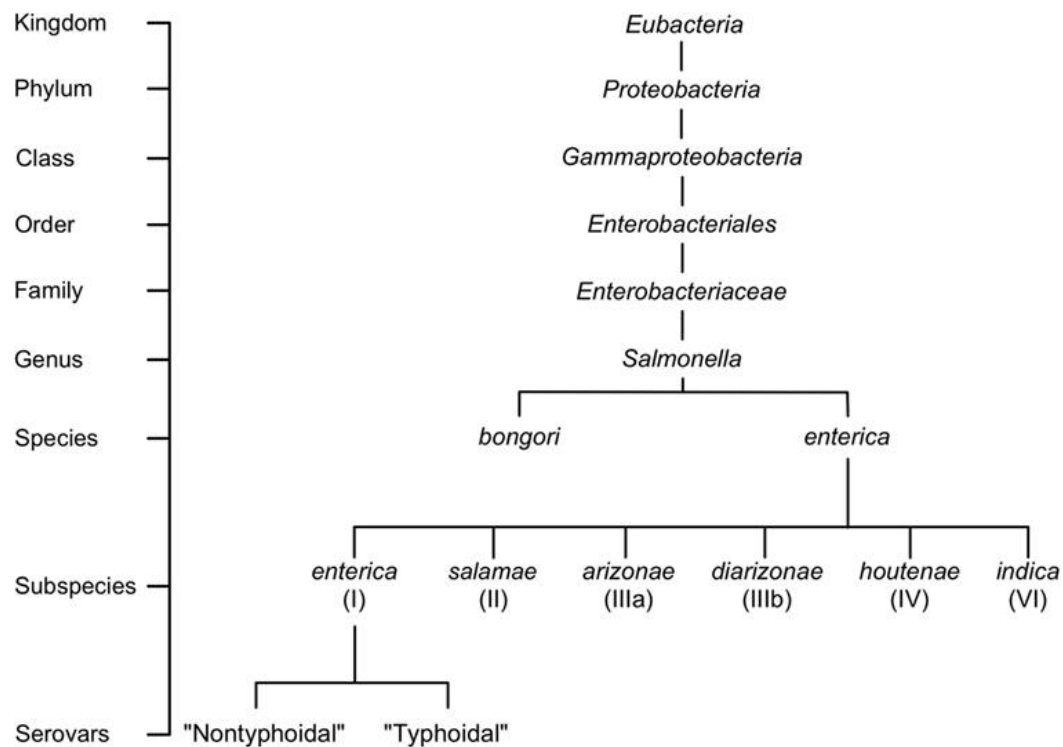


Figure 1. *Salmonella* taxonomy (adapted from MacKenzie et. al., 2017) [10]

Following the Kauffmann-White scheme (as defined and maintained by the World Health Organization Collaborating Center for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France), *Salmonella* is further classified into more than ~2,600 serotypes (serovars)- all of which are regarded as pathogens [9], [11], [12]. Of these, the subspecies *enterica* accounts to about 1,547 serovars, 99% of which may cause infections in animals and humans [12]. In addition, *Salmonella enterica* subsp. *enterica* serovars can further be divided into those that are “typhoidal” and restricted to humans (*i.e.*, serovars Typhi and Paratyphi A) which cause enteric or typhoid fever in humans and those that are “non-typhoidal” and have reservoirs in animals, and cause gastroenteritis in humans [10], [13].

Although vaccination is available to prevent typhoid, it is still estimated that 14.3 million cases occur annually and accounts for 135,900 deaths globally [14]. In the US alone, there are more than 5,000 cases of typhoid fever each year (caused by infection with *S. Typhi*) and about 75% of the cases could be linked with international travel [15].

Nontyphoidal *Salmonella*, on the other hand, frequently colonize the gastrointestinal tracts of livestock and poultry with no effective interventions for prevention of disease in humans [15]. It remains as a prevalent cause of bacterial gastroenteritis in humans, causing 94 million illnesses and 155,000 fatalities per year globally [16]. Moreover, invasive non-typhoidal salmonellosis accounts for 535,000 cases annually causing 77,500 fatalities per year worldwide [17]. In fact it is the second most common gastrointestinal infection in humans (after campylobacteriosis) and is a major cause of foodborne outbreaks in the EU/EEA (EFSA/ECDC report, 2021) [18].

Both *S. Typhimurium* SL1344 and 14028s are common laboratory model strains used in mice to elicit typhoid-like symptoms but causes mild gastroenteritis in humans and both strains are implicated in majority of *Salmonella* cases globally [9]. Furthermore, in epidemiological surveillance reports published by the European Food Safety Authority and the European Centers for Disease Control in 2021, *S. Typhimurium*, monophasic *S. Typhimurium* 1,4,[5],12:i), *S. Enteritidis* and *S. Infantis* are the four leading *Salmonella* strains associated with human salmonellosis in the European Union. These strains are also commonly associated with livestock and are therefore a persistent strain found in the food chain that poses risk to food safety and human health. (EFSA/ECDC report 2021)[19], [20].

Salmonella Paratyphi A is a human host-restricted typhoidal *Salmonella* strain which causes paratyphoid fever. Humans are the main reservoir of typhoidal *Salmonella* strains and causes chronic carriage in 1-4% of the cases [21]. An estimated 11-20 million cases with about 128,000 to 161,000 deaths occur globally, from typhoidal *Salmonella* infections every year [22]. Retrospective studies conducted by Ochiai *et. al.*, (2005) and Verma *et. al.*, (2010) reviewing epidemiological reports for *Salmonella*-related infections both hinted that *S. Paratyphi* A is an emerging strain of *Salmonella* causing enteric fever in Asia and is a growing problem due to its changing multi-drug resistance pattern [23], [24].

Most studies focus on *S. Typhimurium*, however the high prevalence of the strains mentioned, suggests inherent differences in virulence are present among different serovars. For instance, *S. Enteritidis* LA5 encodes several complete *Salmonella* pathogenicity islands, and the large plasmid encodes the *spv* genes [25]. Moreover, *S. Infantis* 119944, an emergent strain originally isolated in Israel harbors a large megaplasmid pESI (~280kb), that confers overall increased virulence to avian and mammalian host cell lines and antimicrobial and environmental stress tolerance [26], [27]. Therefore, these strains were also included in this study.

A meta-analysis conducted in 2019 [9] which investigated the world-wide epidemiology of *Salmonella* serovars in animal-based foods found out that the Typhimurium serovar exhibits a worldwide distribution and is considered as a generalist serovar, which explains its ubiquitous ecological distribution and its high persistence in human Salmonellosis reports across the developed economies *i.e.*, second position in Europe, (EFSA and ECDC, 2018) and third in the United States (CDC, 2018) in human Salmonellosis reports. In an economic perspective, in the United States alone, the US Department of Agriculture calculated that the cost of foodborne illnesses associated with *Salmonella* alone, costs 4.14 billion dollars a year in 2018 both at the agricultural and healthcare sector [28].

Because of their significance to food safety, and the economic and health burden they cause to society [15], investigation into the understanding of *Salmonella* from multiple perspectives continues to be of a significant point of research studies.

B. A closer look at the invasion process of *Salmonella*

Salmonella is significant not just as a persistent hazard to public health and the global food supply chain, but also as an effective model system for understanding the basic mechanisms of bacterial disease [29]. *Salmonella* Typhimurium has long been used as a model organism for genetic research, and a wide range of classical and molecular genetic tools are available to identify and characterize potential *Salmonella* virulence genes. Additionally, the availability of *in vitro* tissue culture and small-animal infection

models facilitates the study of complex interactions between host and pathogen at various stages of infection. Although these models may not perfectly simulate human infection, they provide valuable mechanistic insights to the nature of *Salmonella* pathogenesis and host immunity [29], [30].

Infection with *Salmonella* begins with the ingestion of contaminated food or water. The bacteria traverse the gastrointestinal tract and survives the acidity of the stomach, via an adaptive acid-tolerance response on exposure to low pH. The surviving population of salmonellae that makes it into the small intestine, then must traverse the intestinal mucus layer and compete with the gut microflora to access the intestinal epithelia to which they can adhere via their fimbrial adhesins. Adhesion and invasion of the intestinal epithelium is considered a critical step from which further cellular pathogenesis takes place. Salmonellae then invade and proliferate in epithelial cells by a bacterial-mediated macropinocytosis and exhibit strong preference to specialized antigen-sampling cells called M cells (microfold cells) localized in the small intestine's Peyer's patches and after translocation to the basal side of the epithelium, they can be taken up by macrophages and dendritic cells. Alternatively, they can also move through the intestinal epithelia after being taken up by CD18-expressing phagocytes [6], [31]. Additionally, *Salmonella* is also demonstrated to invade through enterocytes. Moreover, *Salmonella* is also capable of destroying tight junctions *in vitro*, which seal the epithelial cell layer and prevent the paracellular movement of ions, water, and immune cells. Coupled with intestinal inflammatory reactions, these processes eventually result into diarrhea and inflammation characteristic of gastroenteritis. The existence of multiple mechanisms for crossing the intestinal barrier demonstrates the significance of this evolutionary strategy to *Salmonella*'s lifestyle as a pathogen [6][29], [32].

C. *Salmonella* enters host cells via multiple invasion mechanisms

The conventional understanding of how *Salmonella* is known to invade cells is through a process known as *trigger mechanism* whereby *Salmonella* triggers its own uptake via a macropinocytic mechanism (Figure 2). One of the earliest demonstrations and detailed

electron microscopy studies of host cell invasions by *Salmonella* were those done by Takeuchi (1967). From these studies, crucial information descriptive of the sequential events of host cell invasion by *Salmonella* were directly observed by transmission electron microscopy of ileal sections from an infected guinea pig model [33]. This corroborates with much earlier histologic studies performed on the same animal model infected with *Salmonella* [34], and even much earlier histological studies by Florey in 1933 observing early stages of bacterial invasion of the intestinal mucosa [35]. Later on, Galan and Curtis in 1989 were able to demonstrate and characterize the existence of invasins that are necessary for *Salmonella* invasion which would later set the foundation for a “Trigger mechanism” model of *Salmonella* invasion using the Type III secretion system [36].

C.1. Invasion via the trigger-like mechanism

Salmonella Pathogenicity Islands (SPIs) are large gene cassettes that contribute to a particular virulence phenotype and typically expressed at a specific condition during infection of *Salmonella* [37]. In current literature, 21 different *Salmonella* pathogenicity islands (SPIs) have been identified in *Salmonella* [38]. Some strains such as *S. Typhimurium* possess fewer SPIs than others [39]. However, many of the identified PAI-encoded genes have only predicted putative functions with no clear role in *Salmonella* pathogenesis as of current time. *Salmonella* encodes a large Pathogenicity Island integrated within its chromosome (termed as “*Salmonella* Pathogenicity Island 1 or SPI-1) which confers a virulence phenotype to the bacteria [40]. This 40 Kb SPI-1 gene locus carries among others, all the genes required for the biosynthesis of a functional type 3 secretion system apparatus, a number of effector proteins and their chaperones and some regulatory proteins [36][41], [42]. The SPI-1 locus is responsible for encoding several structural proteins that assemble into a protein secretion system which function as a syringe-like nanomachine termed as the Type III secretion system 1 (T3SS-1). The bacterium then uses the protein secretion system to inject effector proteins into its host cell upon contact. Invasion of the host cell is initiated by pathogen binding into the host cell surface which activates the insertion of a translocon into the host cell membrane through its affinity for cholesterol [40].

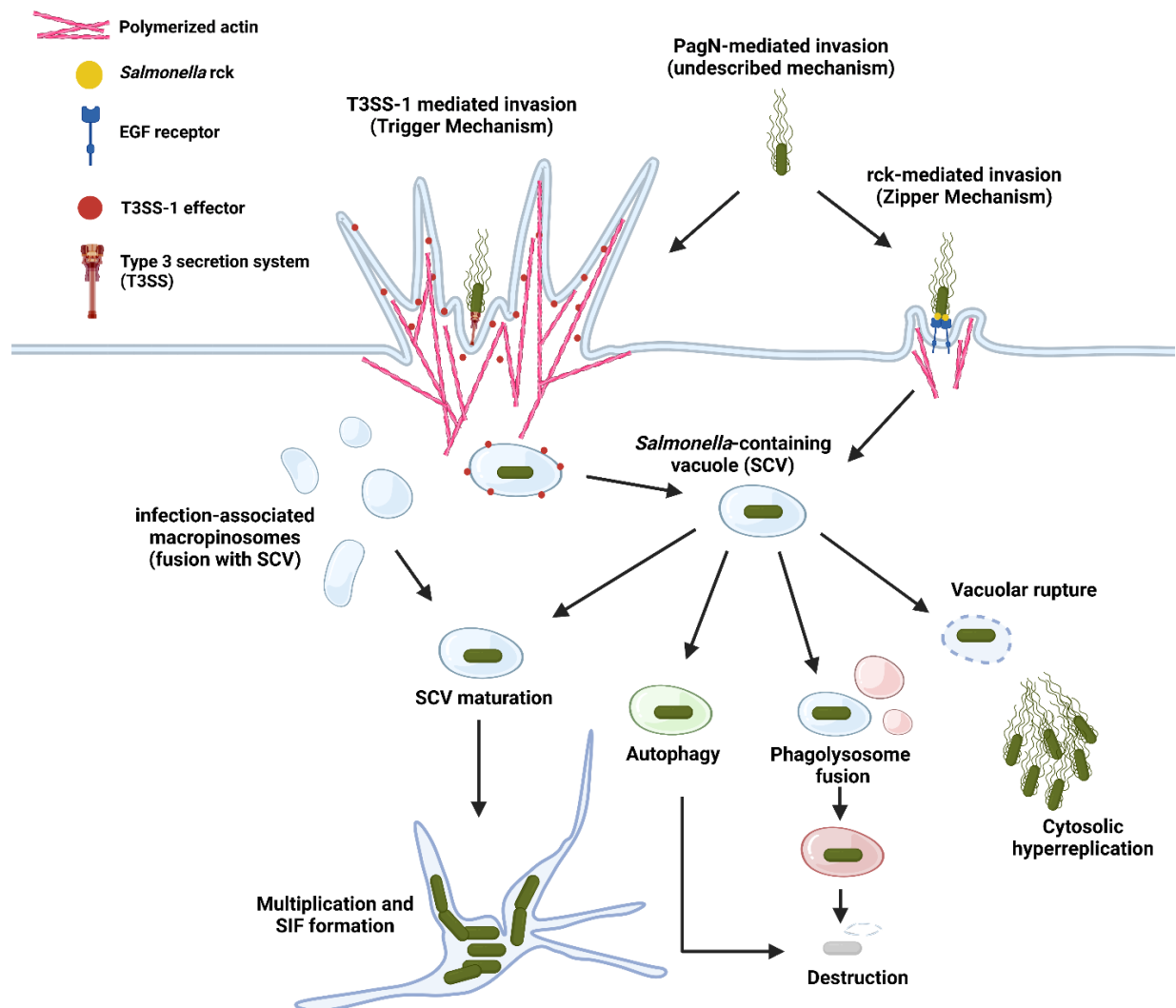


Figure 2. Multiple *Salmonella* entry mechanisms to host cells via “trigger”, “zipper” or PagN-mediated invasion. Image created through Biorender. Adapted from [43], [184]

Translocated effector proteins then mediate various physiological functions into the cell which results into a localized massive intracellular rearrangement of the actin polymers within the entry site of the bacteria and appears as membrane ruffles extracellularly (Illustrated in Figure 2). These membrane curvature structure then allows the bacteria to be enveloped and internalized into the cell eventually confining the bacteria into a vacuole termed as the *Salmonella*-containing vacuole (SCV). This process is

orchestrated in a precise order by several type III secretion effectors which modulate cellular processes to the benefit of the pathogen. At least 15 effectors can be translocated into the host cell by T3SS-1 to induce bacterial entry. Among them, five major *Salmonella* effectors (*i.e.*, SopE, SopE2, SopB, SipA, SipC), drive the manipulation of the cytoskeletal machinery within the host which leads to the internalization of the bacterium into its host cell [43]. The functions of each SPI1 protein subunit are summarized in Table 1.

Table 1. Summary of annotated Type III secretion system 1 (T3SS-1) effectors associated with *Salmonella* invasion into host cells

Effector	Localization	T3SS	Activity	Cellular targets	Pathway/cellular process
AvrA	SPI1	1	Acetyltransferase Deubiquitinase	-catenin, ERK2, IB, MKK4, MKK7, p53	Antiapoptotic, anti-inflammatory
GtgE	Gifsy-2	1/2	Protease		Prevents recruitment of Rab29
PipB2		1/2		KLC (kinesin light chain)	SIF extension
SipA/SspA	SPI1	1		Caspase-3, F-actin, T-plastin	Actin assembly, disruption of tight junctions, PMN migration, SCV positioning
SipB	SPI1	1		Caspase-1	Pyroptosis
SipC/SspC	SPI1	1		Cytokeratin 8, cytokeratin 18, Exo70, F-actin, syntaxin 6	Actin nucleation, SCV maturation
SipD/SspD	SPI1	1			
SlrP		1/2	E3 ubiquitin ligase	ERdj3, Trx	Apoptosis
SopA		1	E3 ubiquitin ligase	Caspase-3, HsRMA1, UbchH7	Invasion, escape from SCV, PMN migration
SopB/SigD	SPI5	1	Phosphoinositide phosphatase/GDI	Cdc24, Cdc42	Invasion, nuclear responses, SCV maturation, fluid secretion
SopD		1/2			Invasion, inflammation, fluid secretion
SopE	SopE	1	GEF	Cdc42, Rab5, Rac1	Actin remodeling, inflammation
SopE2	Phage remnant	1	GEF	Cdc42, Rac1	Actin remodeling, inflammation
SptP	SPI1	1	GAP Tyrosine phosphatase	Cdc42, Rac1, VCP, vimentin	Reversion of actin reorganization, inhibition of ERK activation
SpvC	Plasmid	1/2	Phosphothreonine lyase	ERK2	Inactivation of MAPK
SpvD	Plasmid	1/2			
SspH1	Gifsy-3	1/2	E3 ubiquitin ligase	PKN1	
SspH2	Phage remnant	2	E3 ubiquitin ligase	14-3-3, AIP, BAG2, Bub3, Filamin A, Profilin-1, Sgt1, UbchH5-Ub	
SteA		1/2			
SteE	Gifsy-3	1/2			

Adapted from Ramos-Morales, 2012 [44]

C.2. Invasion via a zipper-like mechanism

In the mid-1990s, an outer membrane protein that confers *Salmonella* a resistance to complement-mediated killing by serum complement proteins was described by independent groups [45], [46], and was thus termed “rck” (*i.e.*, resistance to complement killing). It was then, characterized to be a novel mechanism by which *Salmonella* initiates adhesion and invasion for which a mechanism was not described yet. Rck is a 19 kDa precursor protein encoded by *Salmonella* which is then cleaved and inserted into the outer membrane as a mature 17.4kDa protein, and confers serum resistance- specifically, resistance to complement lysis by preventing C9 polymerization [45], [46].

Rck belongs to the Ail/Lom protein family which consists of several bacterial or phage outer membrane proteins involved in the virulence of Gram-negative pathogens expressing these proteins. They share a common predicted structure of eight transmembrane beta sheets which are conserved among the Ail/Lom protein family and four cell surface-exposed loops which have more sequence variability [46], [47] which may account to a variety of functional characteristics as described by previous studies, such as resistance to complement, adhesion, invasion and bacterial survival in macrophages or host persistence [48]–[50].

Rosselin and colleagues in 2010 identified the domains of rck necessary for adhesion (amino acid residues 140-150) and invasion (amino acid residues 150 -159) and demonstrated that rck-mediated invasion produces microvillus-like membrane extensions akin to a zipper-like invasion mechanism canonically exemplified by *Listeria monocytogenes* [51]. More recently, it was identified by proof-of-principle that epidermal growth factor receptor is required as a direct receptor for Rck-dependent adhesion and invasion of *Salmonella* and that rck-EGFR interaction provide a signaling cascade which leads to internalization of rck-expressing bacteria [52]. Altogether, this opens the paradigm that *Salmonella* can invade cells both by a trigger mechanism via a SPI-1 mediated process and via a zipper-like entry process via the rck membrane protein.

C.3. Other Invasion mechanisms

Although it has been known that *Salmonella* can invade through both a trigger-like mechanism and a zipper-like entry mechanism utilizing SPI-1 effectors and *rck*, respectively [52], there is growing evidence that *Salmonella* utilizes other invasins to internalize into host cells [7], [43].

In studies conducted independently by other research groups, it was also demonstrated that SPI-1 deficient *Salmonella* strains can be internalized into epithelial and fibroblastic cell lines and enter 3D intestinal epithelial cells [53], [54]. Even further, Roche *et. al.*, (2010) demonstrated that *Salmonella*, despite not expressing *invA*, *rck* and *pagN*, can still successfully invade different cell types (albeit in a much less efficient rate), indicating the presence of other invasion factors [55]. Additionally, microscopic examinations of several cell types have demonstrated that these unidentified factors cause membrane and cytoskeletal rearrangements that are comparable to those mediated by either zipper or trigger processes [55]. These findings confirm the isolation of a *Salmonella* strain lacking SPI-1 from a human food-borne disease outbreak in China from a study conducted by Hu *et.al.* in 2008 further strengthening the evidence that *Salmonella* can invade host cells in a SPI-1 independent manner [47], [56].

In a study by Lambert and Smith in 2008, the authors demonstrated that the *Salmonella* outer membrane protein PagN, mediates agglutination of red blood cells, indicating its property as an adhesin [57]. Furthermore, they also demonstrated that by expressing *pagN* in *Escherichia coli*, it mediates adhesion and invasion into mammalian cells in a manner that is dependent on cytoskeletal rearrangements.

Additionally, they also demonstrated that overexpression of PagN in a SPI-1 deficient mutant can partially compensate for the lack of SPI-1. In another study by Fuentes and colleagues (2008), they found that HlyE, a pore forming hemolysin encoded under SPI-18 in *S. Typhi*, promoted invasion in human epithelial cells although the precise

mechanism of invasion remains unknown [58]. Furthermore, when HlyE was heterologously expressed in *S. Typhimurium*, it was demonstrated that it can enhance the colonization into deep organs in mice, which led them to conclude its role as a novel virulence determinant.

These findings show that *Salmonella* can invade host cells via several different mechanisms that are undescribed at the moment. Taken altogether, they open up new perspectives for identification of new invasion factors. Although it is not clear why *Salmonella* serotypes use multiple strategies to invade host cells, Boumart and colleagues (2014), postulated that the development of alternate entry mechanisms mediated by different invasins has broadened the spectrum of host cells which can be invaded by this intracellular pathogen [40], [43].

2. Tetraspanins

Tetraspanins are a large group of a four-pass transmembrane protein ubiquitously expressed in mammalian cells with a wide distribution in the genome of the ancestors of the Metazoa with about 34 members described [59]. They are characterized by a canonical structure of having two extracellular loops where the small extracellular loop (SEL or EC1) is connected to transmembrane domains, TM1 and TM2 and a large extracellular loop (LEL or EC2) which protrude about 3-5nm into the extracellular milieu[60], [61] and is connected via transmembrane domains, TM3 and TM4. In the cytosolic region, a short intracellular loop domain connecting TM2 and TM3 is present, as well as a short intracellular N- and C- terminal tails. Previous authors have investigated each domain to have biological relevance in both physiological and pathological states together with partner tetraspanins and other interacting proteins in the tetraspanin-enriched microdomain [61]–[65]. Owing to their propensity and promiscuity to associate with different partner proteins and other tetraspanins- and to organize and localize them proximally into a platform unit (so-called tetraspanin-enriched microdomains), they are aptly termed as “molecular facilitators”[66], [67]. This property of tetraspanins enable the

formation and stability of specific functional activation and signal transduction complexes [68]–[70] which engage in various aspects of the physiological regulation of cellular development, proliferation, activation, and motility. In addition, they are recently viewed to enhance membrane curvature in certain cellular processes that need it such as in motility/migration, cellular fusion as well as in pathogen uptake [61], [62], [65]. The most well-characterized members of the tetraspanin family include CD81, CD9, CD53, CD82, CD151, CD37 and CD63 [69]–[71]. However, they are also implicated in various pathological states such as in cancer metastasis, infertility, immunity, and infection with a diverse type of microbial pathogens (*i.e.*, viral, bacterial, and eukaryotic). Given the plethora of perceived physiologic and pathologic roles of tetraspanins which are poorly described, this field of study continues to baffle investigators in this area of research.

A. Discovery of tetraspanins

Historically, Atkinson and colleagues, in 1984 were the first to describe the existence of tetraspanins (now re-named CD63) when they identified a monoclonal antibody (mAb) that targets a melanoma-associated antigen [72]. In a follow-up separate work by Oren and colleagues (1990), they first described the existence of the human tetraspanin CD81 (or TAPA-1) when they generated a murine monoclonal antibody that targets a previously undescribed 26-kilodalton cell surface protein, which when blocked at the antigenic epitope, induces a reversible antiproliferative effect on a human lymphoma cell line [73]. Because of such an effect of the antibody, the antigen target was named as TAPA-1, Target of an Antiproliferative Antibody. In another work by Azorsa and colleagues (1991), they further characterized the immunological properties of CD63 [74], and concluded that together with CD81 and other membrane associated proteins sharing a homologous structure, are members of a new family of related molecules which are later termed as *Tetraspanins* [71]. 25 years later, Zimmerman and colleagues in 2016, were able to publish the complete full length crystal structure of human CD81, which by far, is one of the most well studied among the tetraspanin family [61]. With this, several other structural studies have followed that resolved the structures of other tetraspanins such as

that of CD9, CD53, cryo-EM structures of CD9/EWI-2 complex and CD81/CD19 complex and resolution of large extracellular loop of TSPAN15 [75]–[78].

B. General structure of tetraspanins

Tetraspanins are defined by a common structure which is composed of four transmembrane domains (TM1 to TM4) with short intra-cytosolic N- and C-terminal regions, and two extracellular loops termed as EC1 for SEL (short extracellular loop) and EC2 or LEL (large extracellular loop), as illustrated in Figure 3A. While the structural functionality of the SEL is yet to be fully elucidated, the LEL on the other hand has been characterized to have five distinct helical segments, designated as α , β , γ , δ , ϵ - loops (Figure 3B, *designated A-E*) and a conserved CCG motif [64]. More recently, a cholesterol-binding pocket (Figure 3A) which was postulated to regulate the open-closed molecular conformation of the tetraspanin was deduced from molecular modelling studies by Zimmerman and colleagues (2016). The short C-terminal region is implicated to provide a link to intracellular signaling molecules [61], [79].

B.1 Primary interactions to tertiary interactions – formation of tetraspanin-enriched microdomains (TEMs)

Tetraspanin-enriched microdomains (TEMs) are laterally organized homomeric or heteromeric clusters of tetraspanins and other non-homologous proteins which could be in the order of $0.2\mu\text{m}^2$ area as determined by electron microscopy studies (although size and shape vary considerably). Furthermore, advanced imaging methods such as superresolution microscopy determined the tetraspanin cluster size in the range of 100-150 nm, specifically for CD37, CD53, CD81 and CD82 [64], [80].

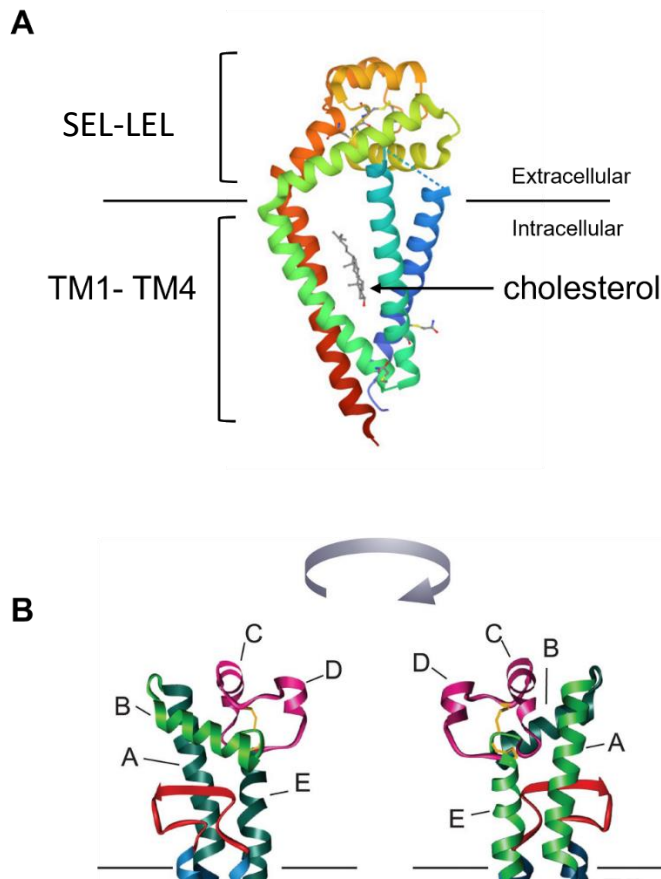


Figure 3. Structure of human tetraspanin CD81 and associated domains (A) adapted from Zimmermann *et. al.*, 2016 (PDB Code 5TCX) illustrating the transmembrane domains TM1 to TM4 (brackets) and cholesterol binding pocket (arrow) and extracellular domains SEL and LEL at the extracellular surface. (B). Two different views of the structure of the tetraspanin CD81 extracellular domains according to the modelling of Seigneuret, 2006 (PDB code 2AVZ) adapted from Charrin *et. al.*, 2009. SEL (or EC1) is in red, structurally conserved domain of the LEL (or EC2) is in green, while the variable region of this LEL in deep pink whereas disulfide bridges of the LEL are in yellow. the different subdomains of the LEL (α , β , γ , δ , ϵ) are labeled A–E. [61], [91], [205]

TEMs are organized into three levels of hierarchy starting from the tetraspanin molecule interacting robustly and directly with its partner protein. This level of interaction is termed as a *primary interaction*. Secondary interactions, on the other hand are those that are only stable under milder solubilization conditions, as that of the many tetraspanin-tetraspanin associations that cross-link primary complexes to large tetraspanin webs. A third level of weak and indirect interactions stabilizes secondary interactions even further. These are based on hydrophobic palmitates that have been palmitoylated and attached

to tetraspanin residues near the inner plasmalemmal leaflet [64], [81]. This level of organization acts as a “molecular facilitator” which assemble lateral clusters of specific proteins into proximity which are needed to exert a physiologic effect such as in cellular activation and transduction processes [70], [82], [83].

Several studies suggests that the role of tetraspanins is related to their ability to interact with other proteins such as adhesion molecule, receptor and co-receptor molecules, major histocompatibility complex antigen, cytoplasmic kinases as well as other tetraspanins [70], [84]–[86]. Insertion into TEMs of these molecules increase the local concentration of these adhesion receptors, facilitating their function as avidity regulators. TEMs have also been shown to play a role in the regulation interaction and crosstalk between different receptors at the plasma membrane, as well as their internalization rate. Moreover, certain signalling pathways are regulated by association with tetraspanins [83], [86]–[88]. Given these properties, TEMs have piqued interest in the field of infection research as potential molecular organizers of receptors for pathogen invasion and trafficking into host cells.

B.2. Importance of tetraspanins in bacterial infections

Evidence from previous studies have already demonstrated that tetraspanins can organize into large networks as tetraspanin-enriched microdomains to interact with partner tetraspanins and localize receptors and adhesion and signalling molecules [89]–[91]. Several pathogens have been demonstrated to hijack these tetraspanin-adhesion platforms to increase the infectivity of various pathogens- including viruses (refer to extensive reviews by van Spriel and Figdor, 2010)[92], bacteria [93]–[95] and parasites [96], [97].

In fact, CD81 has been a prime example of many researchers who have demonstrated its role as a receptor for Hepatitis C virus infectivity [98]–[102]. A recent

review from Karam *et. al.*, (2020) provided an excellent review of the roles of various tetraspanins in bacterial infections, collating the research works performed in the last three decades on the possible roles of tetraspanins in infections involving *Aeromonas hydrophila*, *Burkholderia*, *Coxiella burnetii*, *Corynebacterium diphtheriae*, *Chlamydia*, *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Salmonella Typhimurium* [103]. In so far, among the tetraspanins explored involved in bacterial pathogenicity, only Uroplakins have been demonstrated to be involved directly as adhesion receptor for *E. coli* FimH adhesin binding [104], [105].

Based on previous studies, the LEL domain seem to be the most important for microbial invasion, as it would be the primary contact in the extracellular environment[60], [62], [64], [102], [106]. Hassuna *et. al.*, 2017 demonstrated inhibition of *Salmonella* binding to murine-derived macrophages by targeting the LEL domains of different tetraspanins. More specifically, recombinant truncated LEL-peptides derived from CD63 as well as antibodies that target to the LEL domain of CD63 exhibited the highest inhibition of *Salmonella* uptake [107], [108].

Green *et. al.*, 2011 have also demonstrated that blocking tetraspanins CD9, CD63 and CD151 with each corresponding antibodies, either as a mono- or combinatorial treatment, reduces adherence of *Neisseria meningitidis* to host cells during *in vitro* experiments [94]. Additionally, Ventress *et. al.*, (2016) for example have demonstrated that LEL-derived peptides from tetraspanin CD9 inhibit *Staphylococcus aureus* adherence to keratinocytes [109]. More recently, Elgawidi *et. al.*, and Sangsri *et. al.*, investigated the role of tetraspanins in membrane fusion of cells, resulting to the formation of multi-nucleated giant cell (MNGC) formation induced by *Burkholderia* infection. They found that knocking out CD81, or treatment of cells with CD81 or CD9-derived LEL peptides limit formation and size of MNGC but has no effect in adhesion and internalization of *B. pseudomallei* in infected cells [110], [111].

As the role of tetraspanins in the pathogenesis of microbial infections have become more apparent, there is an emerging interest in designing approaches that target tetraspanins to limit microbial infections. A review by Hemler (2008) and Hassuna (2009) have highlighted proposals for strategies for targeting tetraspanin proteins and concluded that doing so, addresses the potential therapeutic advantages of tetraspanin-based antimicrobial agents since they target the host rather than the microorganisms, decreasing the likelihood of emerging antimicrobial resistance from pathogens [108], [112]. Moreover, because of their broad specificity and low toxicity, it could be an appealing candidate for antimicrobial drug development and further research.

In this project we investigated the role of the host factors, tetraspanins CD81 and CD9 in the context of *Salmonella* infection. By using a combination fluorescence microscopy and tissue culture infection assays, we provided insights into the potential role of these tetraspanins during *Salmonella* invasion and replication. Furthermore, creation of an overexpression of these tetraspanins provided a gain-of-function model while generation of knock-out models confirmed a loss-of-function phenotype in our infection models. We mainly focus on our investigation on *Salmonella* invasion and the contributions of tetraspanins CD81 and CD9 but additionally, we show that CD81 could also affect the long-term survival of *Salmonella* in host epithelial cells. These cellular models for infection will be invaluable to further provide future directions to better understand the mechanistic details of how tetraspanins facilitate and contribute, not only to *Salmonella* invasion but also in its survival and persistence in host cells.

Scope and limitations

To clarify the role of human CD81 in the context of *Salmonella* infection, we investigated how CD81 expression in epithelial cells affects *Salmonella* adhesion, invasion and replication. Moreover, the consequence of perturbing the CD81 structure with known interactors during the infection process was also investigated.

We present herein the findings that were gathered in so far concerning epithelial cell models based on our *in vitro* infection data, microscopy data and mutational experiments. In the future, we further foresee the advancement into a more physiologically relevant system that would allow us to mimic conditions that would closely simulate *in vivo* conditions. This could be achieved through derivation of intestinal organoids as well as mouse lines that may be useful to address the questions relevant to our research.

Objectives of the study

This project seeks to explore and understand uncharacterized mechanisms by which *Salmonella* utilizes different strategies to invade susceptible host cells. Specifically, the role that tetraspanins CD81 and CD9 contribute to this process. We looked further into the intracellular fate of *Salmonella* as influenced by overexpression or loss of expression of CD81. Furthermore, we found a role of CD9 in the infection process and additionally described it herein.

Specifically, we addressed following three objectives:

1. Investigation of the role of CD81 at the early invasion process of *Salmonella* to host cells.
 - a. Effect of overexpression of human CD81 in host cell surface towards susceptibility to *Salmonella* invasion using HepG2 cells
 - b. Effect of deletion of human CD81 in intestinal epithelial cells (*i.e.*, HT29-MTX-E12 and Caco2-BBe1) to *Salmonella* invasion
 - c. Visualization of the recruitment dynamics of CD81 and CD9 during early invasion of *Salmonella* to Caco2-BBe1 cells
2. Investigation on the effects of perturbing CD81 and its effect to the invasion process of *Salmonella* to host cells
 - a. Perturbation of CD81 at the LEL-domain (using anti-CD81 antibodies, recombinant LEL peptides and HCV E2 glycoprotein).
 - b. Cholesterol depletion from cholesterol-binding pocket of CD81 (using methyl-beta-cyclodextrin).
 - c. Stimulation of CD81 activity using epidermal growth factor (EGF) treatment.
3. Demonstration of the extent of involvement of CD81 in the infection process during long-term infection with *Salmonella* in epithelial cells.

Results

1. Investigation of the role of CD81 in early events of *Salmonella* invasion

A. CD81 overexpression increases invasion of non-typhoidal and typhoidal *Salmonella* strains

CD81 has been reported in previous studies to play a role in the pathogenesis of various bacterial pathogens such as *Listeria monocytogenes* [93], *Burkholderia pseudomallei* [111], and *EPEC* [113]. In this project, we were interested to determine the role of CD81 into *Salmonella* invasion. We created an overexpression system by lentiviral transduction to see any effect mediated by the cell surface expression of CD81 in HepG2 cells. HepG2 cells were known to be CD81-deficient [98] and were used as an ideal control to investigate the consequence of CD81 expression. The resulting cells were thereafter validated for surface expression of CD81 by flow cytometry. CD81-negative and CD81-positive single cells were sorted by fluorescence activated cell sorting and further expanded. Figure 4A shows the flow cytometry histogram derived from scatter plots single HepG2 cells sorted after surface staining with APC-conjugated anti-human CD81 antibodies. Human CD81 surface expression is absent on wild-type HepG2 as revealed by flow cytometric analysis (Figure 4A, *gray-region*) whereas HepG2 cells transduced to overexpress hCD81 shows high CD81 surface expression (Figure 4A, *red histogram*). The sorted cells were expanded and were then used for infection experiments with *Salmonella*.

To investigate the role of human CD81 in *Salmonella* invasion, *Salmonella enterica* serovar Typhimurium SL1344 was used for infection experiments (Figure 4B). Intracellular bacteria were recovered and enumerated after infection using a gentamicin killing assay and counts were reflected as a ratio of colonies recovered after cell lysis from total initial inoculum used for infection. Based on results in Figure 4B, overexpression of human CD81 in HepG2 cells increased *Salmonella* invasion significantly.

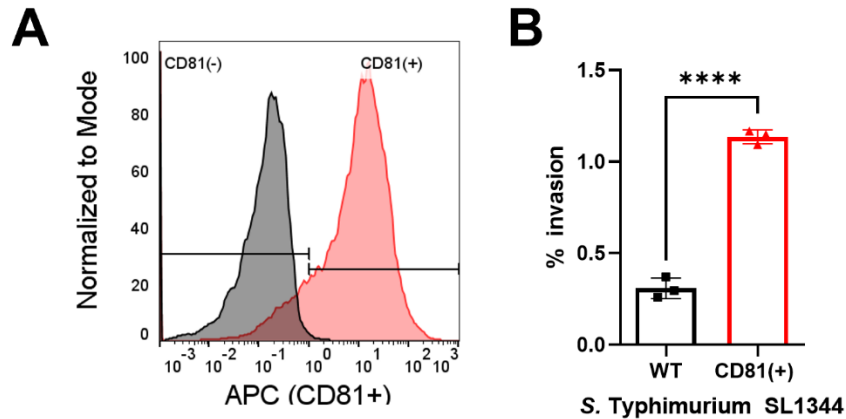


Figure 4. CD81 surface expression in HepG2 correlates with susceptibility for *Salmonella* invasion. (A) Flow cytometry staining profile of wild-type CD81-negative HepG2 (gray region) and CD81-positive HepG2 after transduction with pTrip-hCD81 (red region) to overexpress CD81. (B) Intracellular *Salmonella enterica* serovar Typhimurium SL1344 recovered from wild-type and CD81-overexpressing HepG2 after Gentamicin Killing Assay. % invasion is expressed as a ratio of internalized bacteria recovered after cell lysis from total initial inoculum used for infection (MOI 10:1) 1.5 hrs post-infection. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a Student's t-test, **** $P < 0.0001$.

To test whether this effect is strain-dependent, different representative non-typhoidal and typhoidal *Salmonella* strains were also tested (Fig. 5). *S. Paratyphi* A 45157 (Fig. 5A) was used to represent the typhoidal strain of *Salmonella* whereas, *S. Infantis* 119944 (Fig. 5B), *S. Enteritidis* LA5 (Fig. 5C) and *S. Typhimurium* 14028s (Fig. 5D) were used to represent the non-typhoidal *Salmonella* serovars. In all cases it was observed that the overexpression of human CD81 further enhances *Salmonella* invasion in HepG2 cells compared to when CD81 cell surface expression is absent (wild-type HepG2). This indicates that the effect seen upon CD81 overexpression could be independent of the strain used during infection of HepG2 cells with *Salmonella*. Furthermore, it was also noted that among all the strains tested, *S. Infantis* 119944 and *S. Enteritidis* LA5 invaded better into HepG2 cells compared to *S. Typhimurium* SL1344 or *S. Typhimurium* 14028s or *S. Paratyphi* A 45157. It should be noted that due to the lower invasiveness of *S. Tm* 14028s, infection time for this bacterium in these experiments were prolonged to 60 minutes (and a corresponding gentamicin treatment of 1.5 hours as well).

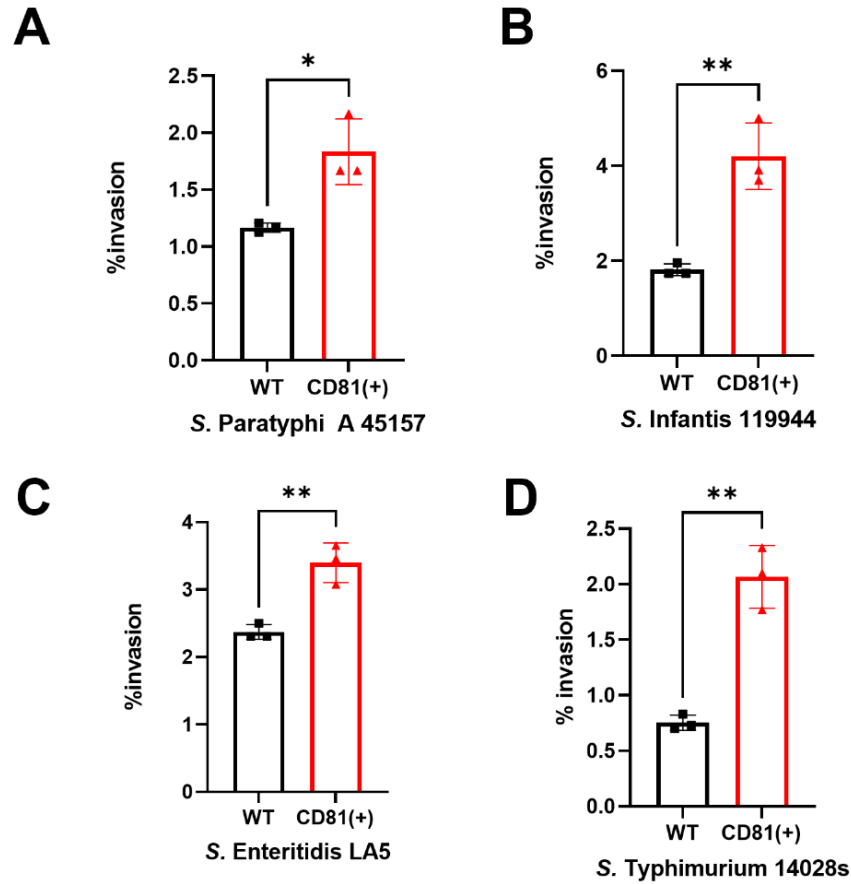


Figure 5. Invasion of various *Salmonella* strains is CD81-dependent. Gentamicin Killing Assay of *S. Paratyphi* A 45157 (A), *S. Infantis* 119944 (B) and *S. Enteritidis* LA5 (C) and *S. Typhimurium* 14028s (D). Infection with (A-C) were incubated for 30 minutes with HepG2 cells, while (D) was incubated for 1 hour (to account for low invasion efficiency) prior to Gentamicin Killing Assay. % Invasion is expressed as a ratio of internalized bacteria recovered after cell lysis from total initial inoculum used for infection (MOI 10:1) post- gentamicin treatment of infected cells. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a Student's t-test, * $P < 0.05$, ** $P < 0.01$.

Since the effect we observed was seen across different serovars of *Salmonella*, we further wanted to investigate whether this effect is specific under SPI-1 inducing conditions. A previously reported triple mutant deficient of all known invasins of *Salmonella*, $3\Delta(\Delta invA, \Delta pagN, \Delta rck)$ was known to retain invasive activity in certain cells, albeit an undescribed mechanism (Roche *et. al.*, 2018)[114]. Using this triple mutant, we observed that CD81 expression in HepG2 cells enhances *Salmonella* association after 60

minutes of infection (Fig. 6A). Furthermore, we evaluated a 3Δ mutant complemented with *rck* and found that zipper-like invasion of *Salmonella* is likewise enhanced in the presence of CD81 on the cell surface of host cells (Fig. 6B). This led us to believe that CD81 association during *Salmonella* invasion is perhaps a general mechanism involved in the uptake and internalization of *Salmonella* and is not exclusively linked to a SPI-1 mediated process.

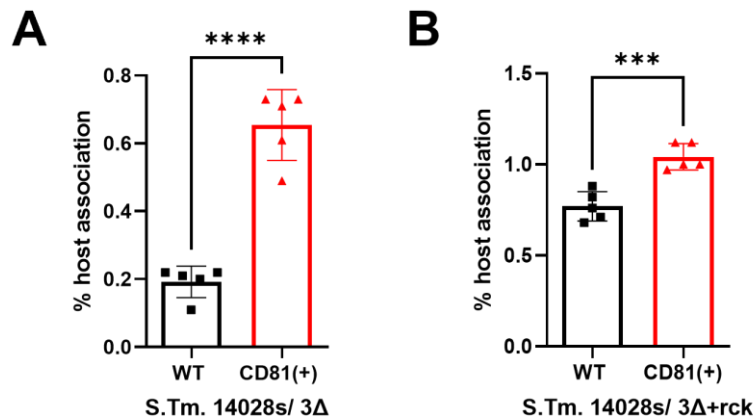


Figure 6. CD81-mediated *Salmonella* infection susceptibility of epithelial cells is SPI-1 independent. (A) Gentamicin killing assay of *Salmonella* Typhimurium 14028s mutant invalidated for three currently known invasion factors, 3Δ (*invA*, *pagN*, *rck*); and (B) *rck*-complemented 3Δ mutant, associate with host cells in a CD81-dependent manner. Bacteria were incubated for 1 hour (MOI 100:1) with HepG2 cells following tissue culture infection protocol. %host-association (adherent and internalized bacteria) is expressed as a ratio of all colony counts recovered after cell lysis from total initial inoculum used for infection. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a Student's t-test where ***P<0.001 or ****P<0.0001.

B. Human CD81 and human CD9 overexpression influences *Salmonella* invasion

We further investigated whether other tetraspanins play a role in *Salmonella* invasion, as tetraspanins are known to associate with each other upon formation of a tetraspanin-enriched microdomain [70], [90]. Thus, we constructed HepG2 cells overexpressing either rat CD81 or human CD9. A summary of the flow cytometric staining

histogram of different tetraspanin overexpression (derived from scatter plot of single cells gated for positive staining with the anti-tetraspanin antibody of interest) is shown in Figure 7A. Wildtype HepG2 cells (gray histogram) were transduced to overexpress either human CD81 (red histogram), human CD9 (green histogram) or rat CD81 (blue histogram). HepG2 cells with high surface expression of the tetraspanin of interest were sorted and then further expanded prior to further experiments. These transgenic cell lines were then infected with *S. Typhimurium* SL1344 to see the effect of different tetraspanin overexpression towards *Salmonella* invasion (Figure 7B). HepG2 cells were seeded in 24-well plates and were infected with log-phase *S. Typhimurium* SL1344 after which gentamicin killing assay is performed. Intracellular salmonellae were recovered and enumerated 1.5 hours post-infection and the effect of the different tetraspanin overexpression is represented as a measure of % invasion of *S. Typhimurium* SL1344. Overexpression of human CD81 and CD9 enhanced *Salmonella* invasion, whereas overexpression of rat CD81 did not lead to any appreciable increase in *Salmonella* invasion (compared to wild-type HepG2 cells, Figure 7B). This may suggest a biological role for human tetraspanin CD81 in the context of *Salmonella* invasion of cells and not for rat CD81 ectopically expressed in human epithelial cells. Additionally, our results also indicate that apart from human CD81, overexpression of human CD9 also serves to enhance *Salmonella* invasion in CD81-deficient HepG2 cells.

C. *Salmonella* invasion can be attenuated by targeting human tetraspanin CD81

To determine the specificity of CD81 influence towards *Salmonella* invasion, we investigated the effects of using different reagents that could possibly block or enhance CD81 activity in the host cells prior to infection with *Salmonella*. The use of antibodies against tetraspanins, as well as recombinant truncated LEL-derived peptides have been

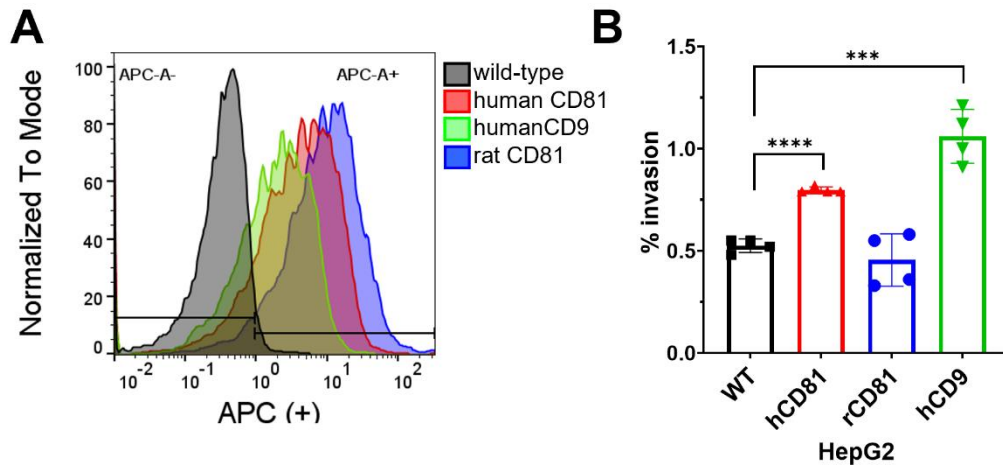


Figure 7. Overexpression of human CD81 and human CD9 influences *Salmonella* invasion in epithelial cells. (A) Flow cytometry staining histogram profile of HepG2 cells post-transduction after overexpressing various tetraspanins. Wild-type HepG2 cells lack endogenous expression of CD81 but not CD9. (B) Overexpression of human CD81 or human CD9 influences invasion of *S. Typhimurium* SL1344 1.5 hours post-infection at an MOI 10:1. % invasion is expressed as a ratio of internalized bacteria recovered after cell lysis from total initial inoculum used for infection. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a Student's t-test where *** $P < 0.001$ or **** $P < 0.0001$.

reported by previous studies to induce tetraspanin-specific effects towards host cells during infection with different bacterial pathogens [95], [107], [109]–[111].

Towards this direction, we used CD81-derived LEL peptides to perturb *Salmonella* interactions with CD81. A previous report by Hassuna *et. al.* (2017) noted that the use of recombinant CD81-LEL derived peptide inhibited macrophage uptake of *S. Typhimurium* [107]. We were interested to see whether this effect could be replicated in HepG2 (Figure 8). Additionally, the intestinal epithelial cell line Caco2-BBe1 expresses CD81 (Figures 13A and 15A), and the effect of blocking CD81 with CD81-LEL peptides in this cell line was also investigated (Figure 9). Briefly, host cells were treated with the 20 μ g/ml of CD81-LEL peptides one hour prior to infection with *S. Tm.* SL1344 followed by gentamicin killing assay. Cell lysates were then plated in LB medium to enumerate recovered host cell-associated bacteria 30 mins post infection (prior to gentamicin treatment) as well as

internalized bacteria, 1.5 hrs post-infection (after gentamicin treatment). Based on results, there was no reduction of *Salmonella* numbers observed upon CD81-LEL peptide treatment compared to mock (medium only) controls (Fig. 8 A-D; Fig 9, A and B).

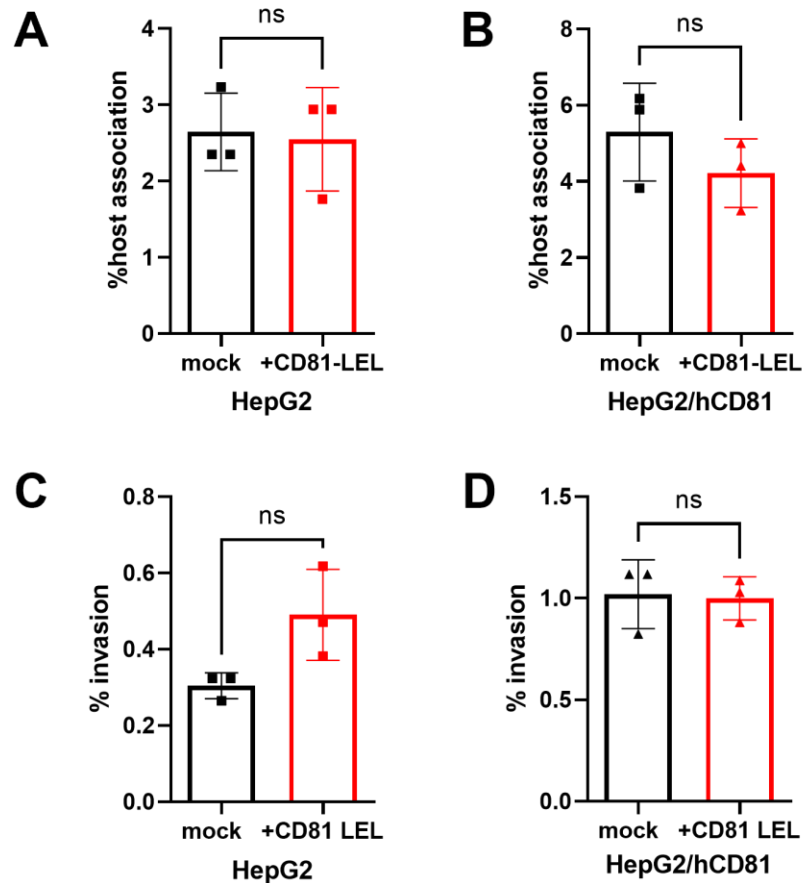


Figure 8. Recombinant CD81-LEL peptide treatment has no effect on *Salmonella* invasion in HepG2 cells. HepG2 (A and C) and HepG2/hCD81 (B and D) were treated with 20μg/ml CD81-LEL peptides 1 hour prior to *S. Typhimurium* SL1344 infection. Cells were infected with bacteria at an MOI of 10:1 for 30 minutes. Adherent and internalized bacteria in proportion to the inoculum used are reflected as %host-association (A and B), whereas internalized bacteria relative to the inoculum is reflected as %invasion (C and D) after 1 hour gentamicin treatment (100μg/ml) to kill extracellular salmonellae. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a Student's t-test where ns, not significant.

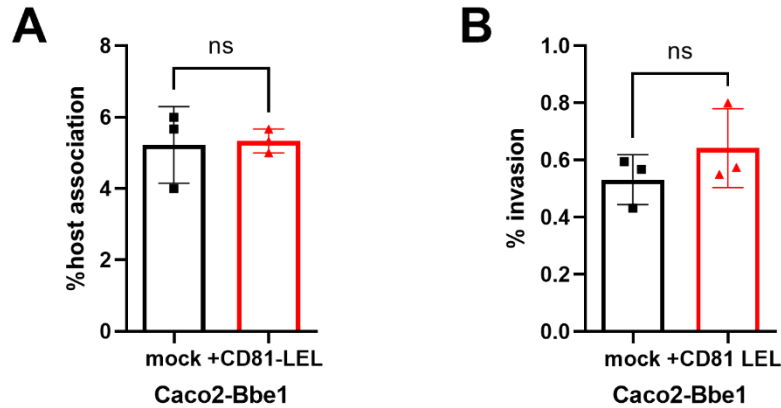


Figure 9. Recombinant CD81-LEL peptide treatment has no effect on *Salmonella* invasion in Caco2-BBe1 cells. Recombinant CD81-LEL treatment, 20µg/ml of Caco2-BBe1 cells 1 hour prior to *S. Typhimurium* SL1344 infection. Cells were infected with bacteria at an MOI of 10:1 for 30 minutes. (A) Adherent and internalized bacteria in proportion to the inoculum used are reflected as %host-association, whereas (B) internalized bacteria relative to the inoculum is reflected as %invasion. Data are the representative mean +/- S.D. of at least three independent experiments and were compared using a Student's t-test where ns is not significant.

In all cases, *neither* the level of host-associated bacteria (30 mins post-infection) prior to gentamicin treatment nor the level of internalized salmonellae recovered after gentamicin treatment (1.5 hours post-infection) differed significantly in the number of salmonellae recovered in the cell lysates. As we expected an inhibitory effect on *Salmonella* invasion upon CD81-LEL treatment, the specificity for CD81-mediated *Salmonella* invasion remained unclear.

Next, we investigated the effect of using an anti-CD81 antibody that targets the LEL domain [100], [115], [116]. Anti-CD81 antibodies were used to block CD81 antigenic epitope to see whether this might affect *Salmonella* internalization in HT29-MTX-E12 cells. HT29-MTX-E12 cells are CD81-expressing (Figure 14A) mucus-producing intestinal epithelial cells which can be differentiated to exhibit a goblet-cell like phenotype [117], [118]. Briefly, host cells were treated with anti-CD81 antibodies or its corresponding

isotype control at a concentration of 10µg/ml for 1 hour at 37°C prior to infection with *S. Typhimurium* SL1344. Antibodies were also titrated by 2-fold dilution series to see if reducing the concentration of the antibodies in the medium prior to infection decreases the potency of treatment with CD81 specific antibodies in relation to *Salmonella* host association (Figure 10). In so far observed with HT29-MTX-E12 cells, a reduction of *Salmonella* associated with host cells was observed at a concentration of 10µg/ml. Reductions in bacterial association with the host (adhered and internalized) were also seen at a concentration of 5µg/ml and at 2.5µg/ml but not as significant compared to 10µg/ml. These data show that anti-CD81 treatment exerts an inhibitory effect towards CD81-dependent *Salmonella* invasion in intestinal epithelial cells.

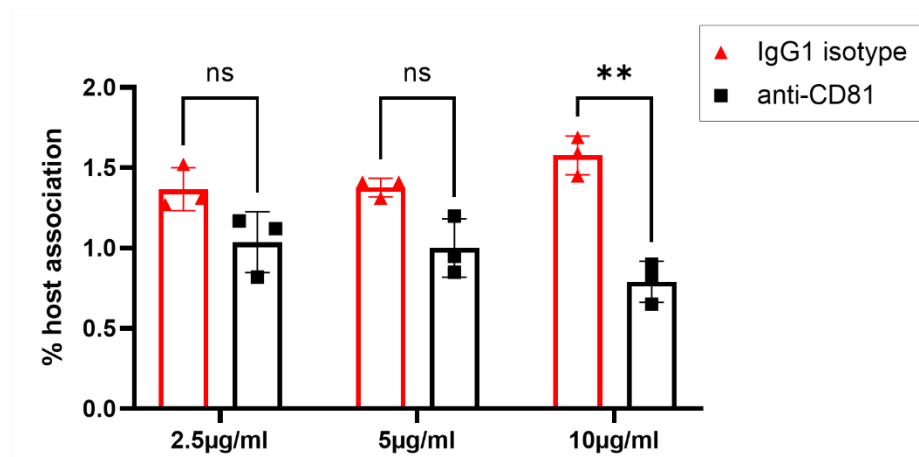


Figure 10. Anti-CD81 treatment of HT29-MTX-E12 cells reduces *Salmonella* invasion versus its IgG1 isotype control at various concentrations for 1 hour at 37°C, 5% CO₂ prior to infection with *S. Typhimurium* SL1344. Cells were infected with bacteria at an MOI of 10:1 for 30 minutes. Adherent and internalized bacteria in proportion to the inoculum used were reflected as % host-association. Data are the representative mean +/- S.D. of at least three independent experiments and were compared using a Student's t-test where ns, not significant, and **P < 0.01.

The host tetraspanin CD81 is known to possess a cholesterol-binding pocket in its central cavity and is known to dictate its structural conformation into an open- or closed-type configuration [61]. This further affects secondary interactions with other ligands [65],

[119] that may associate within the tetraspanin-enriched microdomain (TEM). Furthermore, previous studies have found that membrane cholesterol depletion plays a role in the invasion of different pathogens such as *Listeria monocytogenes*, *Salmonella*, and *Plasmodium* [97], [120]–[122].

Methyl beta-cyclodextrin (M β CD) acts to sequester cholesterol in cell membranes perturbing the cholesterol balance in the inner and outer leaflet of the host cell membrane [123], [124]. It was therefore tested whether cholesterol depletion using an agent such as M β CD could potentially perturb the CD81-mediated enhancement of *Salmonella* association with host enterocytes. To this end, a CD81-knockout cell line of Caco2-BBe1 was generated to compare CD81-specific effect (Figure 15A) during cholesterol sequestration. It was observed that treatment of both CD81(+) Caco2-BBe1 cells resulted in reduction of *Salmonella* invasion in both cases compared to control treatment (Figure 11). More specifically, we observed that CD81(+) cells, upon cholesterol depletion, led to a significantly greater reduction of *Salmonella* burden internalized into cells. CD81-deficient cells are still negatively impacted in terms of *Salmonella* uptake but is much less so, than CD81(+) cells upon M β CD treatment.

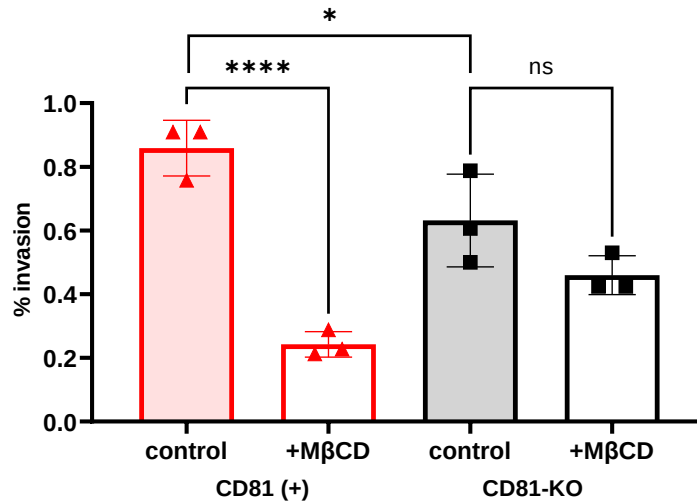


Figure 11. Cell surface cholesterol depletion impacts CD81-dependent *Salmonella* invasion in Caco2-BBe1 cells. Cell surface cholesterol depletion with 10mM methyl- β -cyclodextrin (M β CD) treatment, 15 minutes at 37°C, 5% CO₂ prior to infection for 30 minutes (MOI10:1) and Gentamicin Killing Assay. Internalized bacteria relative to the inoculum is reflected as %invasion. Data are the representative mean $\pm$ S.D. of a two independent experiments and were compared using ANOVA followed by post-hoc multiple comparison test with the mock control where ns, not significant, *P<0.05, ****P<0.0001.

HCV E2 glycoprotein is a known interactor of CD81 [102], [125], [126]. Following this premise, we sought to utilize this knowledge to test whether a direct binding of a known ligand for CD81 perturbs the influence of CD81 to *Salmonella* invasion in HepG2 cells. Increasing concentrations of HCV E2 glycoprotein were added to HepG2 cells one hour prior to infection with *S. Typhimurium* SL1344 (MOI 10:1) and a gentamicin killing assay experiment was conducted to recover intracellular bacteria which is represented as the ratio of the recovered bacterial counts from the cell lysates to the initial inoculum used.

Based on the results from Figure 12, increasing concentrations of HCV E2 from 0.1 μ M, 1 μ M to 2 μ M appear to have a dose-dependent effect on reducing *Salmonella* invasion on HepG2 cells overexpressing CD81 (red bars). Interestingly, no effect on *Salmonella* uptake was seen on wild-type HepG2 cells (black bars) treated with HCV E2 prior to infection, which suggests that this effect is specific to the expression of CD81. Mock controls where no HCV E2 was added to the infection medium results in a similar trend observed when CD81 is overexpressed in HepG2 cells resulting to a significant

increase in *Salmonella* uptake. Such a dose-dependent reduction of *Salmonella* uptake from HCV E2 treatment could possibly be attributed to the saturation of HCV E2 binding to CD81 which then perturbs the organization of CD81 in a tetraspanin-enriched microdomain.

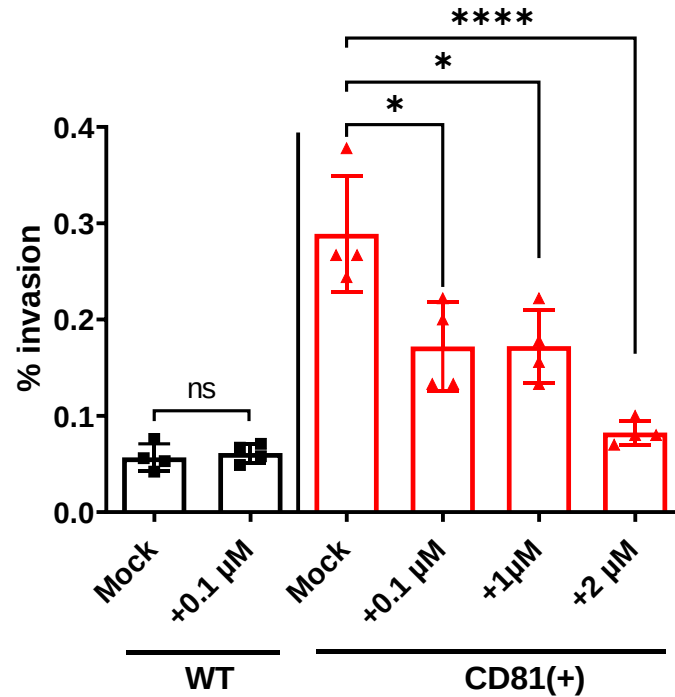


Figure 12. CD81-influenced *Salmonella* invasion can be blocked with recombinant HCV E2 in a dose-dependent manner. Wild-type HepG2 (black bars) and HepG2/hCD81 (red bars) were infected with *S. Tm* SL1344 for 30 mins at an MOI 10:1 followed by a gentamicin killing assay to enumerate internalized bacteria. % invasion is expressed as a ratio of internalized bacteria recovered after cell lysis from total initial inoculum used for infection. Data are the representative mean $\pm$ S.D. and were tested using one-way ANOVA followed by post-hoc multiple comparison test with the mock control where ns, not significant, * $P < 0.05$ or **** $P < 0.0001$.

D. CD81 is localized in cellular protrusions involving f-actin remodeling

It is known that addition of soluble growth factors, such as epidermal growth factor (EGF) can induce membrane ruffle formation and cytoskeletal rearrangements via rac1 activation [127], [128], akin to a *Salmonella* invasion induced membrane ruffle formation (albeit a more global effect throughout the cell rather than being localized to a *Salmonella* entry site). Therefore, we tested whether CD81 could localize into these membrane ruffles.

To this end, Caco2-BBe1 cells were serum-starved for 16 hours in culture medium devoid of fetal calf serum to deplete the active pool of Rac1-GTP [129], [130]. Cell culture medium was then replaced by the same medium containing 100ng/ml of EGF for 10 minutes followed by PFA fixation and staining for actin and CD81 and host cell nuclei. Based on the results obtained, unstimulated cells contain randomly distributed clusters of CD81-staining specks on the cell surface which tend to also be more proximal perinuclearly to the host cell nuclei (Figure 13A). As expected, upon EGF stimulation, CD81 seemed to have redistributed along host cellular protrusions along the edges (Figure 13B in *yellow arrows*) which are also sites of active actin polymerization and membrane remodelling implying that CD81 on the cell surface actively rearranges into distinct cluster compartments dependent on a stimulus provided. Furthermore, CD81 is observed to localize in what seemed to be fused vesicle structures (*white arrows*), although this still need to be further verified and will not be covered in this project.

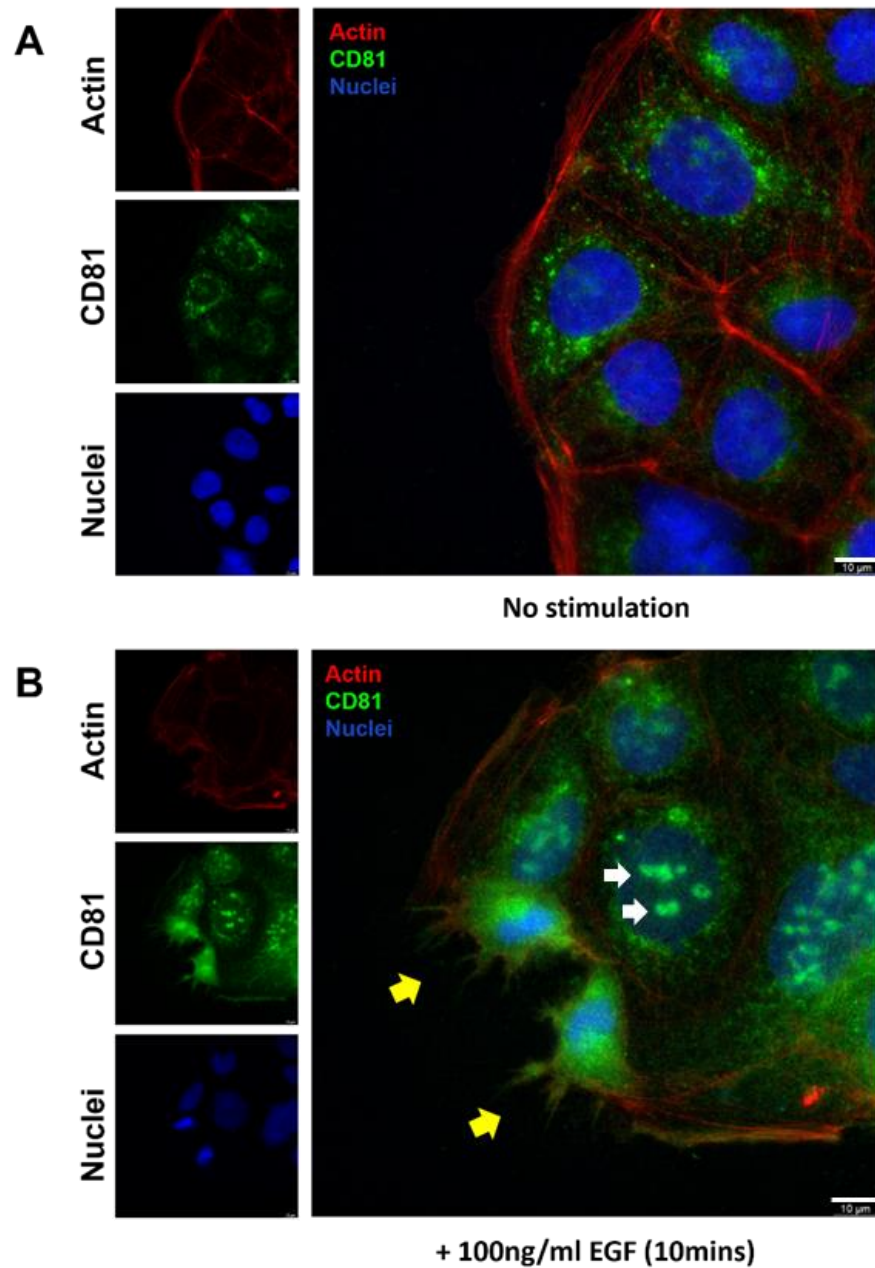


Figure 13. Epidermal growth factor (EGF) stimulation drives CD81 localization into cellular motility structures. (A) Distribution of CD81 in cell surface of Caco2-BBe1 under normal culture conditions. (B) Stimulation of Caco2-BBe1 with 100 ng/ml EGF for 10 minutes. CD81 distributes along actin polymerization sites along the protruding edges of the cell (yellow arrows) and into vesicle-like formations. Scale bare 10μm.

E. Deletion of CD81 in intestinal epithelial cells attenuate *S.Typhimurium* invasion

The Caco2 cell line is one of the most commonly used models of the small intestine employed in the study of intestinal pathogens[131]–[134] which can be differentiated to express enterocyte-like features similar to those found in the small intestine, such as formation of a microvilli brush border, tight junctions and many enterocytic enzymes and transporters among others [135], [136]. HT29-MTX-E12 is a stable homogenous human colonic epithelial cell line derived from the HT29 cultures after treatment with methotrexate [137]. When differentiated, HT29-MTX cells have a goblet cell-like phenotype and secrete MUC2 mucins which are predominantly expressed in the large intestine. Similarly, this cell line has been used as to model infection processes associated with intestinal pathogens [138]–[140] Due to these properties, both cellular models have been valuable models for investigating gut microbial infections [141], [142].

We knocked out CD81 from both cell lines using a pLentiCRISPRv2 encoding a CD81-targeting synthetic guide RNA which was packaged into a lentiviral vector and transduced to HT29-MTX E2 and Caco2-BBe1 cells to create CD81-deficient intestinal epithelial cell line models for *Salmonella* infection experiments (refer to Materials and Methods for details).

Figure 14A, shows the flow cytometry staining histogram of CD81 (+) and CD81-deficient, HT29-MTX-E12 cells (in red and gray histogram peaks, respectively) after CRISPR/Cas9 knock-out of CD81 and selective enrichment for CD81(+) and CD81(-) cell populations through FACS. Following enrichment and expansion, cells were then used for gentamicin killing assay. As anticipated, results show that deletion of CD81 in HT29-MTX-E12 cells also led to less efficient invasion of *Salmonella* to host cells (Figure 14B).

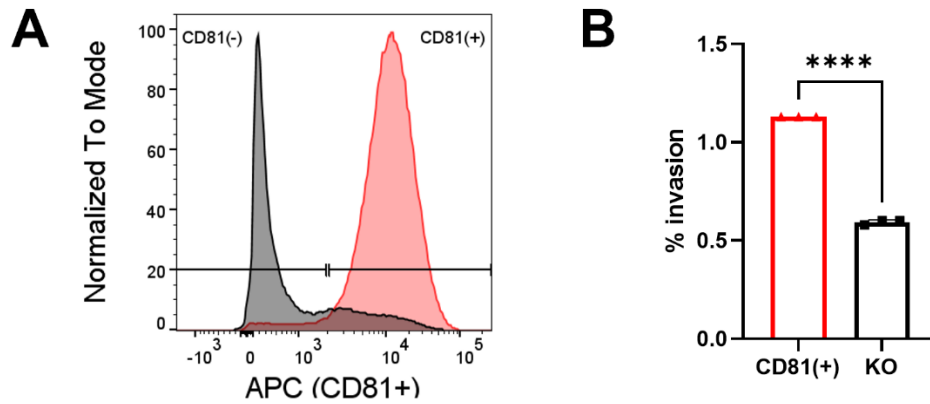


Figure 14. Deletion of CD81 in HT29-MTX-E12 cells attenuates *Salmonella* invasion (A) flow cytometry staining profile of CD81 (red histogram) in HT29 MTX E12 cells. CD81-knockout cell population is represented in grey histogram. (B) Gentamicin killing assay of HT29-MTX-E12 cells expressing CD81 (red bar) or knocked out for CD81 expression (black bar). %invasion is calculated as a ratio of the recovered internalized bacteria to the initial inoculum after Gentamicin Killing Assay. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a Student's t-test, where **** $P < 0.0001$.

Likewise, CRISPR/Cas9-mediated deletion of human CD81 was also performed on Caco2-BBe1 cells to model the effect of deleting human CD81 in an enterocyte model of human cells upon infection with *Salmonella*. Figure 15A shows the flow cytometry staining histogram of human CD81 in CD81-negative single cells (*gray histogram*) and CD81-positive single cells of Caco2-BBe1 (*red histogram*), respectively. As in the case of HT29-MTX-E12 cells, deletion of CD81 led to a significant decrease of *Salmonella* invasion in Caco2-BBe1 cells (Figure 15B). CD81 expression has been previously reported to positively influence *Listeria monocytogenes* invasion [93]. Therefore, we used *L. monocytogenes* EGD to recapitulate the functionality of our knockout model with this previous study.

Caco2-BBe1 cells were similarly infected with *L. monocytogenes* EGD at an MOI of 10:1. Gentamicin killing assay results show that a reduction in bacterial uptake was also observed (Figure 15C) as with the prior observations made with *Salmonella* invasion

experiments previously. This implies further that other invasive enteric pathogens utilizing a zipper-like mode of entry can exploit cell surface CD81 to invade host cells.

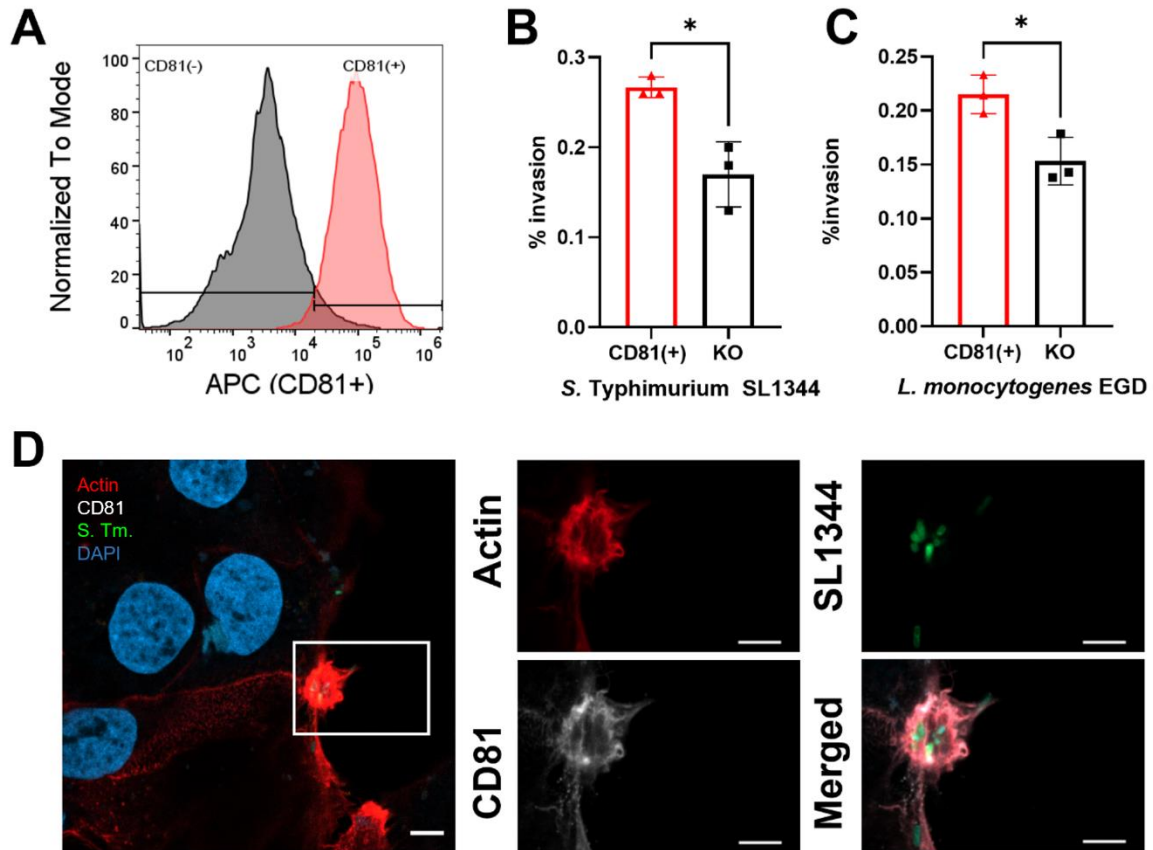


Figure 15. Deletion of CD81 in Caco2-BBe1 cells attenuates *Salmonella* invasion (A) Flow cytometry staining of Caco2-BBe1 cells, CD81-KO population (gray) and CD81-positive population (red). Gentamicin killing assay of (B) *S. Typhimurium* SL1344 and (C) *Listeria monocytogenes* EGD in Caco2-BBe1 cells (CD81-positive vs CD81-KO). %Invasion is calculated as a ratio recovered internalized bacteria to the initial inoculum during infection. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a student's t-test $*p < 0.05$ (D) Laser scanning confocal imaging of Caco2-BBe1 cells 15 mins post-infection with *S. Tm.* SL1344. Magnified subsection is membrane ruffle initiated by early invading bacteria with CD81 recruitment along an area with actin polymerization. Scale bar 5 μ m. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a Student's t-test, $*P < 0.05$.

F. CD81 localization to the *S. Typhimurium* invasion foci requires active uptake of the bacteria and is not a consequence of *Salmonella* adhesion to the cell surface

To visualize the spatio-temporal localization of CD81 in Caco2-BBe1 and its possible recruitment along *Salmonella* invasion foci, we performed an infection experiment using GFP-expressing *S. Typhimurium* SL1344 followed by immunostaining for CD81, f-actin and host nuclei (Figure 15D). Using laser confocal microscopy, we visualized membrane ruffles during early *Salmonella* invasion as indicated by the localized presence of cytoskeletal rearrangements and *Salmonella* cells (Figure 15D, white box). Localization of CD81 is apparent in membrane ruffles (enriched in f-actin pooling) associated with *Salmonella* entry sites. This confirms that CD81 facilitates early *Salmonella* invasion by locally reorganizing CD81 in the host membrane during invasion of host cells.

To test whether CD81 could localize at adhesion sites of *Salmonella*, Caco2-BBe1 cells were infected with GFP-expressing *S. Typhimurium* SL1344 at an MOI of 100:1 and immediately kept at 4°C for 60 minutes to inhibit bacterial uptake but allow *Salmonella* to bind to host cells. Upon plating, no observable difference was noted with CD81(+) Caco2-BBe1 compared to its CD81-KO equivalent, indicating that CD81 does not influence *Salmonella* adhesion (Figure 16 A).

We further complemented these observations with imaging experiments and found that CD81 does not seem to contribute to *Salmonella* adhesion when cellular uptake of *Salmonella* is inhibited at low temperature because of its marked absence in the *Salmonella* adhesion site (Figure 16B, white box inset). This further confirm the results with colony counts obtained in Figure 16A where a difference in *Salmonella* adhesion between the wild-type cell line expressing endogenous levels of CD81 versus its knockout counterpart is no longer apparent suggesting that an active uptake of *Salmonella* drives CD81 localization to *Salmonella* entry site, either exerted by the host cell or the bacteria itself or a concerted process of both.

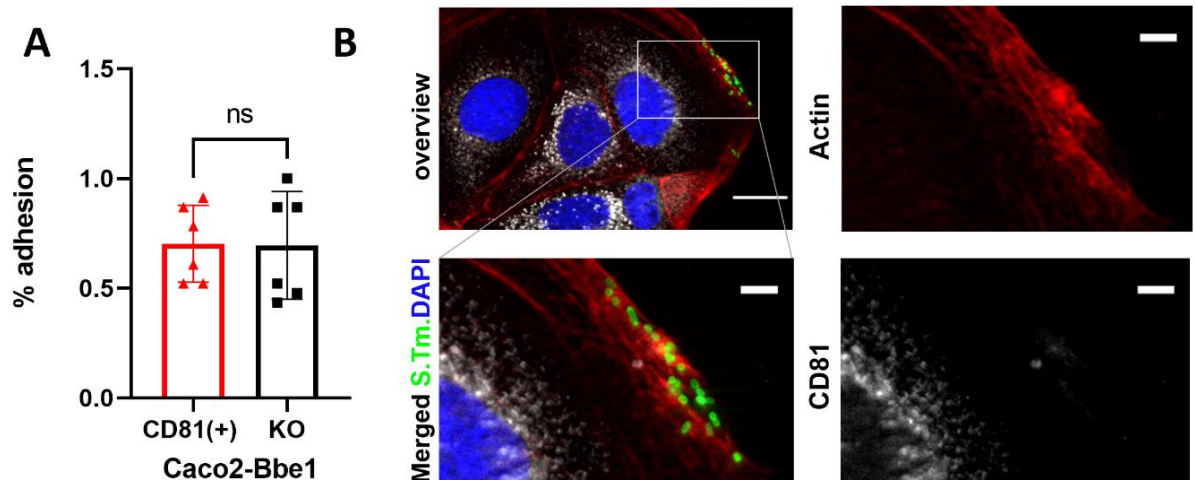


Figure 16. CD81 does not localize in *Salmonella* adhesion sites (A) Adhesion assay of *S. Typhimurium* SL1344 (MOI100, 60 mins, 4°C). (B) *Upper left panel.* Immunostaining of infected cells from (A) for CD81 (white) GFP-expressing *S. Typhimurium* SL1344 (green) actin (red) and host nuclei/DAPI (blue) Scale bar 20µm. *Lower left panel.* Cropped and magnified image of adherent salmonella on Caco2-BBe1 cell surface. CD81 (white, *lower right panel*) is located more perinuclear and does not colocalize with cortical actin (red, *lower right panel*). Scale bar 5µm. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a Student's t-test, where ns, not significant.

2. Investigation on the contribution of CD9 to *Salmonella* infection dynamics

A. CD9 overexpression in HepG2 increases *Salmonella* invasion but not adhesion

We noted from our HepG2 infection experiments that human CD9 overexpression increase *Salmonella* invasion (Figure 7B) independent of the expression of CD81. Compared to CD81, which is not expressed in HepG2 cells, endogenous expression of CD9 is present and performing lentiviral transduction to integrate human CD9 further

enhanced its overexpression. We also noted that the CD81 overexpression system also had elevated gene expression of CD9 compared to wild type (Figure 17A).

Using the same HepG2 models, we compared the influence of CD9 overexpression on *Salmonella* adhesion and invasion (Figure 17, B and C). For adhesion, we infected HepG2 cells with *S. Typhimurium* SL1344 at an MOI of 50:1 and suppressed bacterial uptake by incubating the infected cell cultures at 4°C for 60 minutes followed by lysis and plating to recover and enumerate bacterial colonies. Based on the results from our adhesion assay, CD9 overexpression seemed to have little to no effect on *Salmonella* adhesion into HepG2 cells as the CFU counts recovered were not significantly different from the wild type HepG2 cells (Figure 17 B). However, internalized bacteria recovered 1.5 hours post infection at 37 °C (Figure 17C), showed that CD9 overexpression significantly increased *Salmonella* invasion in HepG2 cells.

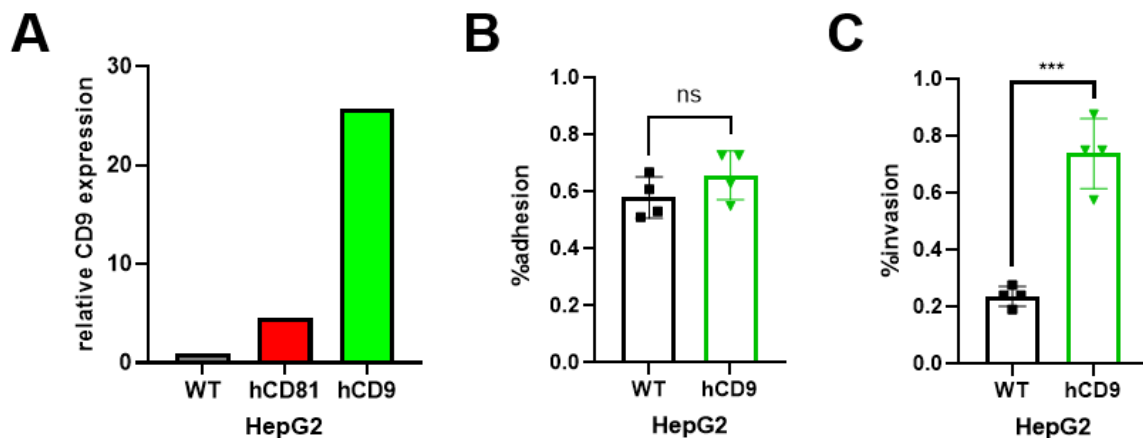


Figure 17. CD9 overexpression favors *Salmonella* invasion into HepG2 cells. (A) relative gene expression of CD9 in wildtype HepG2 (gray), HepG2/hCD81 (red) and HepG2/hCD9 (green). (B) Adhesion assay of *S. Typhimurium* SL1344 (MOI100, 60 mins, 4°C) in wild-type HepG2 cells and HepG2/hCD9 (C) Human CD9 overexpression influences *S. Typhimurium* invasion in HepG2 cells. Data are the representative mean $\pm$ S.D. of at least three independent experiments and were compared using a Student's t-test, where ns, not significant, and ***P<0.001.

B. CD9-LEL peptide treatment does not affect *Salmonella* host association nor invasion in Caco2-Bbe1

We further sought to check whether the use of recombinant CD9-LEL peptides would have an effect in *Salmonella* infection in a Caco2-BBe1 model intestinal epithelial cell model. Cells were pre-treated with CD9-LEL for 1 hour prior to infection which was proceeded by a gentamicin killing assay. The degree of host association of *Salmonella* was examined 30 minutes post-infection before gentamicin treatment, and we did not see any difference in mock-treated wells compared to that of wells pre-treated with recombinant CD9-LEL peptides. Internalized bacteria were also enumerated post gentamicin treatment and, in both treatments, show no significant difference between the mock-treated and CD9-LEL treated wells infected with *Salmonella* (Figure 18) which do not corroborate with earlier experiments which used CD9-LEL peptides to reduce *Salmonella* adhesion and uptake in macrophages [107].

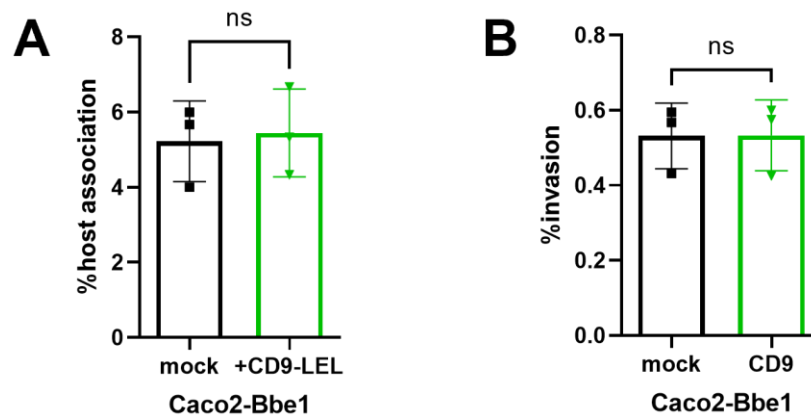


Figure 18. Recombinant CD9-LEL treatment of Caco2-BBe1 cells has no effect on *Salmonella* invasion. Caco2-BBe1 cells were treated with 20µg/ml of CD9-LEL peptides 1 hour prior to *S. Typhimurium* SL1344 infection (MOI10:1) for 30 minutes. (A) Adherent and internalized bacteria in proportion to the inoculum used are reflected as %host-association, whereas (B) internalized bacteria relative to the inoculum (after Gentamicin killing assay) is reflected as %invasion. Data are the representative mean $\pm$ S.D. and were compared using a student's t-test where ns, not significant.

C. Human CD9 is trafficked along *Salmonella* entry sites during infection

Next, we were interested with the spatio-temporal localization of CD9 during early *Salmonella* invasion and to address this, we investigated its trafficking in Caco2-BBe1 cells 15 minutes post-infection (Figure 19) by laser confocal microscopy imaging. Using GFP-expressing *S. Typhimurium* SL1344, we infected Caco2-BBe1 cells and fixed and immune-stained for CD9, f-actin and host nuclei.

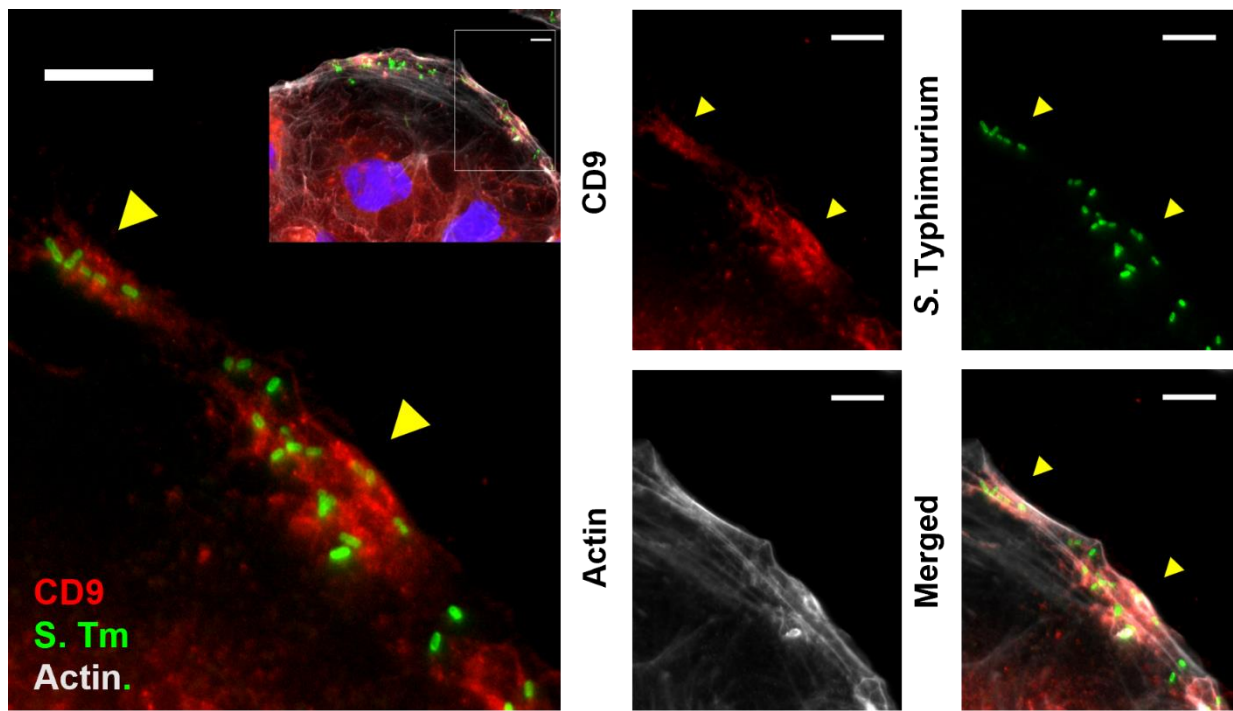


Figure 19. Human CD9 localizes to *Salmonella* entry site during invasion. Human CD9 (red) is recruited along *Salmonella* entry sites (yellow arrows) during early invasion of Caco2-BBe1 cells, 15 mins post-infection at MOI 100:1. *Upper right box inset* Overview of infected cells. Scale bar 10µm

Immunostaining of CD9 in Caco2-BBe1 cells infected with *Salmonella* at an MOI 100 for 15 minutes also seemed to localize CD9 in *Salmonella* entry sites where active *Salmonella* invasion is happening. Surprisingly, CD9 seemed to reorganize at *Salmonella* invasion foci (Figure 19, *yellow arrows*) with cortical actin (Figure 19, *merged frame*). Furthermore, we noted no apparent membrane ruffles where CD9 is clustered around

Salmonella cells. This was strikingly different compared to our previous findings with CD81 localization during *Salmonella* invasion (Figure 15D).

Together with our gentamicin killing assay and adhesion assay data, this further suggests that CD9 influences *Salmonella* invasion and facilitates the active uptake of the bacteria into host cells, perhaps being recruited to the *Salmonella* invasion foci earlier than CD81 due to the absence of imminent formation of any cytoskeletal reorganization and membrane ruffling.

D. CD9 and CD81 are co-recruited during *Salmonella* invasion

Previous reports have noted that CD9 and CD81 are tetraspanin partners frequently found together in the tetraspanin-enriched microdomain [143], [144]. As both CD81 and CD9 seem to possibly influence *Salmonella* invasion independently in our HepG2 model, we sought to investigate the spatial dynamics of CD9 and CD81 localization at the cellular level. To this end, Caco2-BBe1 cells were found to be the most appropriate model for imaging studies as it was found easier to observe membrane ruffling on the cell edges where *Salmonella* initiates adhesion and invasion. Caco2-Bbe1 cells were infected with GFP-expressing *S. Typhimurium* SL1344 at an MOI of 100:1 for 15 minutes and was fixed with 4% PFA followed by double immunostaining with anti-CD9 and anti-CD81 antibodies (Figure 20).

CD9 and CD81 were visualized localizing together in *Salmonella* invasion foci at very close proximity (Figure 20, white arrows). Again, we noted that CD9 seemed to be trafficked towards the invasion foci more readily than CD81 due to its marked abundance around the invading *Salmonella* compared to CD81. This complements our observations (with single tetraspanin immunostaining) in Figure 19, where CD9 localization is not marked by distinguishable f-actin enrichment and membrane ruffling or protrusions, compared to those we observed earlier with that of CD81 in Figure 15D.

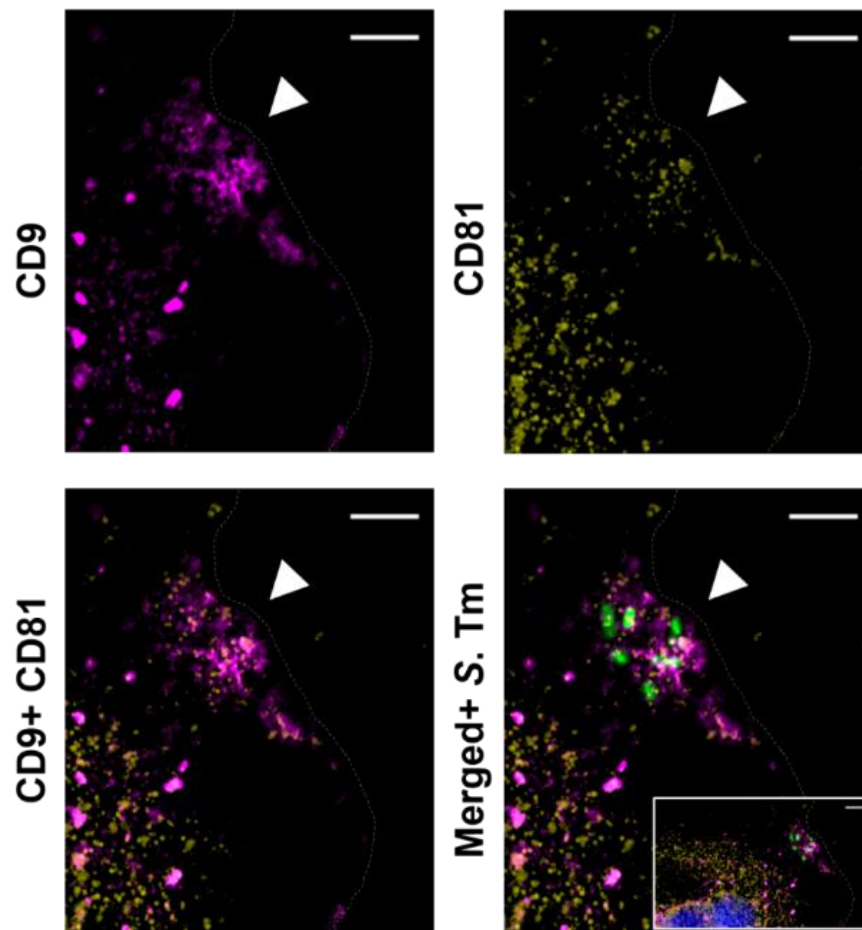


Figure 20. CD81 and CD9 are both recruited at the *Salmonella* entry site during invasion. Double immunostaining of human CD9 (violet, *upper left panel*) and human CD81 (yellow, *upper right panel*). CD9/CD81 overlay (*lower left panel*) on cell surface of Caco2-BBe1 cells during early invasion of *S. Typhimurium* SL1344 (green, *lower right panel*) 15 mins post-infection at MOI 100:1. White dashed line traces cell boundary. *Inset on lower right.* overview of infected host cells where nuclei are visible through DAPI staining (blue). Scale bar 5µm.

To further clarify whether CD9 and CD81 are indeed recruited together as partner proteins during *Salmonella* invasion in Caco2-BBe1, we performed an *in situ* proximity ligation assay, PLA (Figure 21) after immunolabelling CD9 and CD81 in Caco2-BBe1 cells, 15 minutes post-infection with GFP-expressing *S. Typhimurium* SL1344 (MOI 100:1). PLA detects proteins that are at most 40nm apart, indicating not only proximity but possible protein-protein interaction [145].

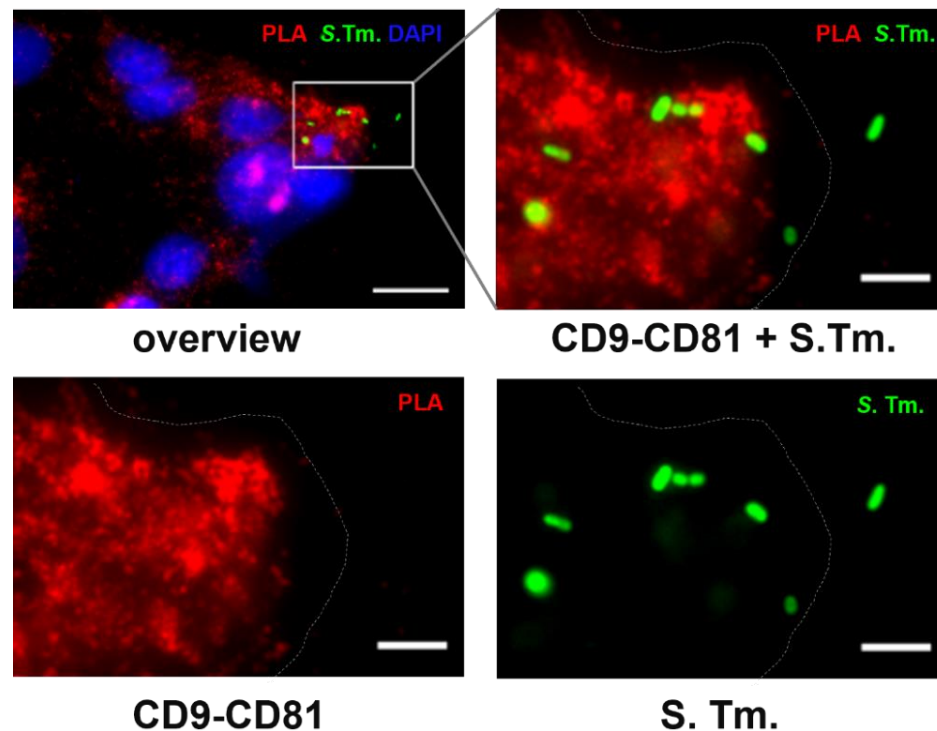


Figure 21. *In situ* Proximity ligation assay confirms CD81 and CD9 co-recruitment to *Salmonella* entry site during invasion of Caco2-BBe1 cells. *In situ* Proximity ligation assay signal (red) from CD9 and CD81 co-localization on host cell surface of Caco2-BBe1 cells during early invasion of *S. Typhimurium* SL1344 (green) validates their co-recruitment and putative protein-protein interaction during *Salmonella* entry. Scale bar 5µm. White dashed line traces cell boundary. *Upper left panel.* Overview of infected host cells where nuclei are visible through DAPI staining (blue). Scale bar 20µm

As shown in Figure 21, PLA signal (red) probing CD9/CD81 interaction were observed to be clustered near the entry point of *Salmonella* during invasion. This observation agrees with previous images from single staining of either CD81 (Figure 15D) and CD9 (Figure 19). Furthermore, this data confirms the previous observation from double immunostaining using anti-CD81 and anti-CD9 antibodies (Figure 20) where we observed that both CD9 and CD81 localizes together at the *Salmonella* invasion foci.

Overall, the *in situ* proximity ligation assay signal was found to be specific for detecting a complex of CD9 and CD81 (Figure 22) since the single antibodies of neither CD81 nor CD9 alone, did not produce any specific PLA signal (red). The control set up with no antibodies also gave no fluorescence signal which validates further that PLA signals were specific from the close interaction of both CD9 and CD81 and their putative protein-protein interaction.

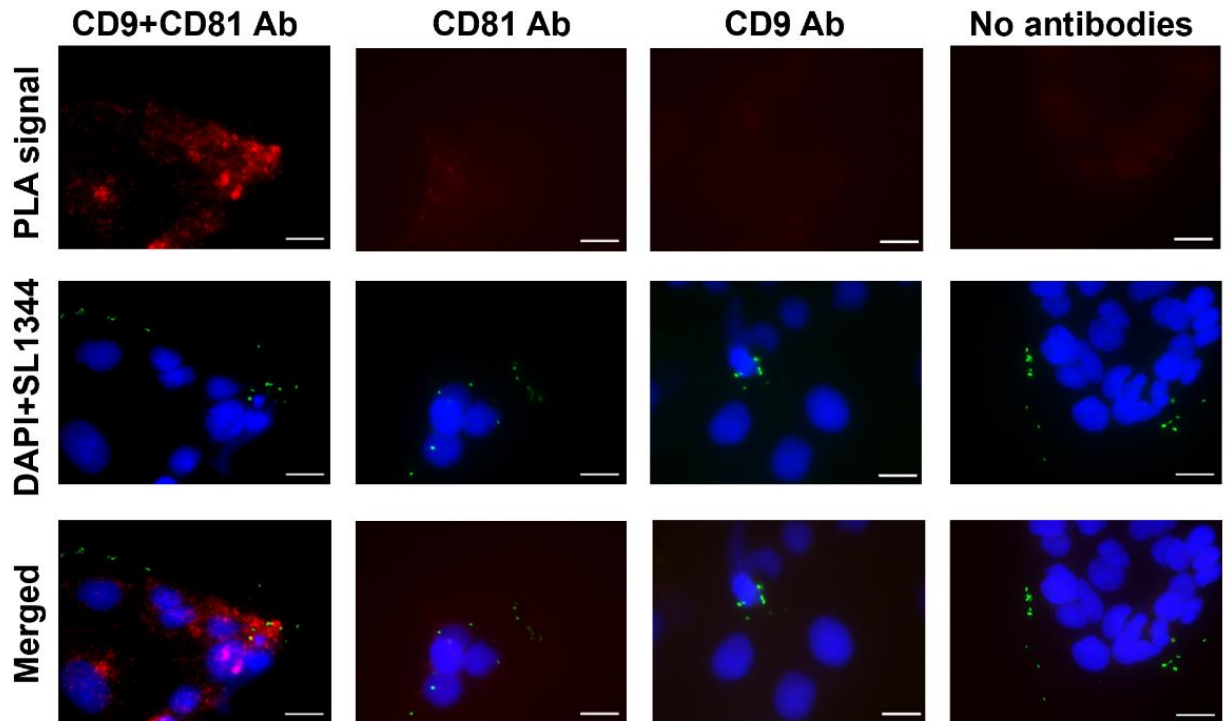


Figure 22. Validation of *in situ* Proximity Ligation Assay of CD81/CD9 co-recruitment at the *Salmonella* entry site. Control set-ups (anti-CD81 only, anti-CD9 only or no antibodies) validating specificity of PLA signal to co-recruitment of CD9 and CD81 interaction during early invasion of *Salmonella* indicating its proximity and suggesting possible protein-protein interaction. Absence of, or addition only of single antibodies to CD9 and CD81 do not produce PLA signal Scale bar 20µm.

3. Investigation on the influence of CD81 expression on epithelial host cells towards long term infection with *Salmonella*.

A. CD81 expression influences the course of *Salmonella* replication in host cells

We investigated the fate of replicating salmonellae inside host cells using our established cellular models for infection. To do this, gentamicin killing assay was performed in infected host cells at an MOI 10:1 then at 1.5 hours post-infection, the gentamicin concentration in the medium was reduced from 100µg/ml to 10µg/ml and the infected host cells were incubated further up to 24 hours up to which, the host cells were lysed and the intracellular bacteria were enumerated by dilution and plating in LB agar plates. Replication ratio was determined as a ratio of the total CFU/well recovered from the 24 hour time point to that of the CFU/well recovered at 1.5 hour time point. This provides an index of the efficiency of replication of *Salmonella* inside the host cells from the time of invasion to its replication within a 24 hour timeframe. As shown in Figure 23A, overexpression of either CD81 (*red bar*) or CD9 (*green bar*) in HepG2 led to a more efficient replication of salmonellae inside host cells 24 hours post-infection. Both CD81 and CD9 overexpressing HepG2 cells achieved more than double the replication efficiency of *Salmonella* compared to that of the wild type HepG2 cells (*black bar*) nor the HepG2 cells expressing rat CD81 (*blue bar*). Furthermore, we observed that *Salmonella* replicates in the same efficiency in HepG2 cells overexpressing rat CD81 compared to that of wild type HepG2, which might suggest that the effects seen from overexpressing human tetraspanins CD81 and CD9 is species- specific and that overexpression of rat CD81 in HepG2 plays no effect in *Salmonella* replication.

On the other hand, it was also observed that *Salmonella* replicates much more efficiently in HT29-MTX-E12 cells rather than HepG2 cells when CD81 is expressed in both cells (Figure 23B). Furthermore, deletion of CD81 in HT29-MTX-E12 (*black bar*) cells led to a less efficient replication of *Salmonella* in host cells 24 hours post-infection which validates the inefficiency of *Salmonella* replication in CD81-deficient wild-type HepG2 cells.

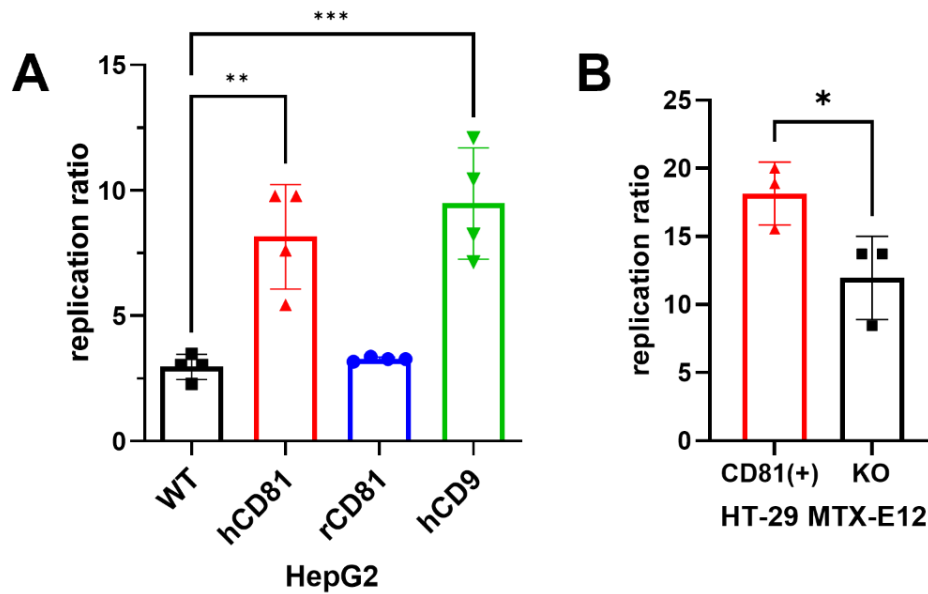


Figure 23. *Salmonella* replication in epithelial cells is influenced by the expression of human CD81 and CD9. (A) Overexpression of human CD81 and human CD9 but not rat CD81 enhances the replication ratio of *Salmonella enterica* serovar Typhimurium SL1344 in HepG2 cells. Wild-type HepG2 is CD81-negative. (B) Loss of CD81 expression in HT29-MTX-E12 cells lead to a reduction of *Salmonella* intracellular replication. Data are the representative mean $\pm$ S.D. and were compared using a Student's t-test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

B. Deletion of CD81 influences subcellular localization of *Salmonella* in Caco2-BBe1 cells

We performed the same assays to evaluate the replication ratio of *Salmonella* in our Caco2-BBe1 model (Figure 24A) and surprisingly, we observed an opposite trend. Deletion of CD81 in this cell line led to far more efficient replication of salmonellae (*black bar*) compared to wild-type CD81-expressing Caco2-BBe1 (*red bar*) by two-fold difference.

Due to the discrepancy that we observed in using Caco2-BBe1 in comparison to both HepG2 and HT29 -MTX-E12 infection models, we sought to further investigate these observations by complementary experiments. A chloroquine resistance assay (Figure 24B) was conducted to determine the proportion of cytosolic *Salmonella* in Caco2-BBe1

cells upon 24 hours post infection. Addition of chloroquine (a lysosomotropic agent), and gentamicin in the same medium has been demonstrated to selectively kill SCV-bound *Salmonella*, as previously reported by Knodler *et. al.*[146]. Briefly, a replication assay is performed as mentioned earlier and at 24-hour timepoint, spent growth medium is replaced with chloroquine (800µg/ml) for 1 hour. To recover intracellular salmonellae, cells are lysed and the lysates are diluted and plated in LB plates to enumerate colony forming units.

We found that by comparison, CD81(+) Caco2-BBe1 cell line harbors far less cytosolic *Salmonella* than CD81-deficient cells (Figure 24B), suggesting that deletion of CD81 in these cells influences the maintenance of salmonellae in the *Salmonella*-containing vacuole, whereby salmonellae preferentially end up in the cytosolic niche and hyperreplicating in the cytosolic compartment rather than maintaining itself in an SCV-bound state.

To confirm this, we tried to visualize the nature of growth of salmonellae inside host cells 24 hours post-infection (Figure 24E and 24F). To do this, we infected CD81(+) and CD81-deficient Caco2-BBe1 (MOI 100:1) with a *Salmonella* reporter strain expressing dsRed constitutively, and an inducible GFP expression once it escapes out of the SCV and hyperreplicates into the cytosol (Figure 24D and 24F, yellow arrow). Infected cells were maintained in 10µg/ml gentamicin-containing culture medium and were fixed and stained as per protocol prior to fluorescence microscopy. Upon observation, we visualized *Salmonella* growing as either cytosolic hyperreplicating clusters (Figure 24D, upper row) or as non-septated filaments of bacteria, some of which can reach more than 20µm in length (Figure 24D, lower row, 24E yellow arrows). We noticed that in infected CD81(+) cells, the incidence of *Salmonella* exhibiting a filamentous growth, seemed much more frequent. We enumerated the filamentous clusters per field of observation and indeed, found that the tendency of *Salmonella* to form non-septated filamentous morphology is strikingly higher in CD81(+) Caco2-BBe1 cells compared to CD81-deficient Caco2-BBe1 cells (Figure 24C). Furthermore, based on microscopy observations, hyperreplicating clusters were confirmed to be more distributed in the cytosol based on the level of expression intensity of GFP (Figure 24D, upper row, GFP panel). Long *Salmonella*

filament clusters were more distributed in the SCV due to a weaker expression of GFP signal. Altogether, these results suggest that CD81 expression not only influences *Salmonella* invasion of host cells, but also the long-term infection process, whereby *Salmonella* fails to maintain itself in an SCV-bound state in the absence of CD81 expression in Caco2-BBe1 as an enterocyte model of infection.

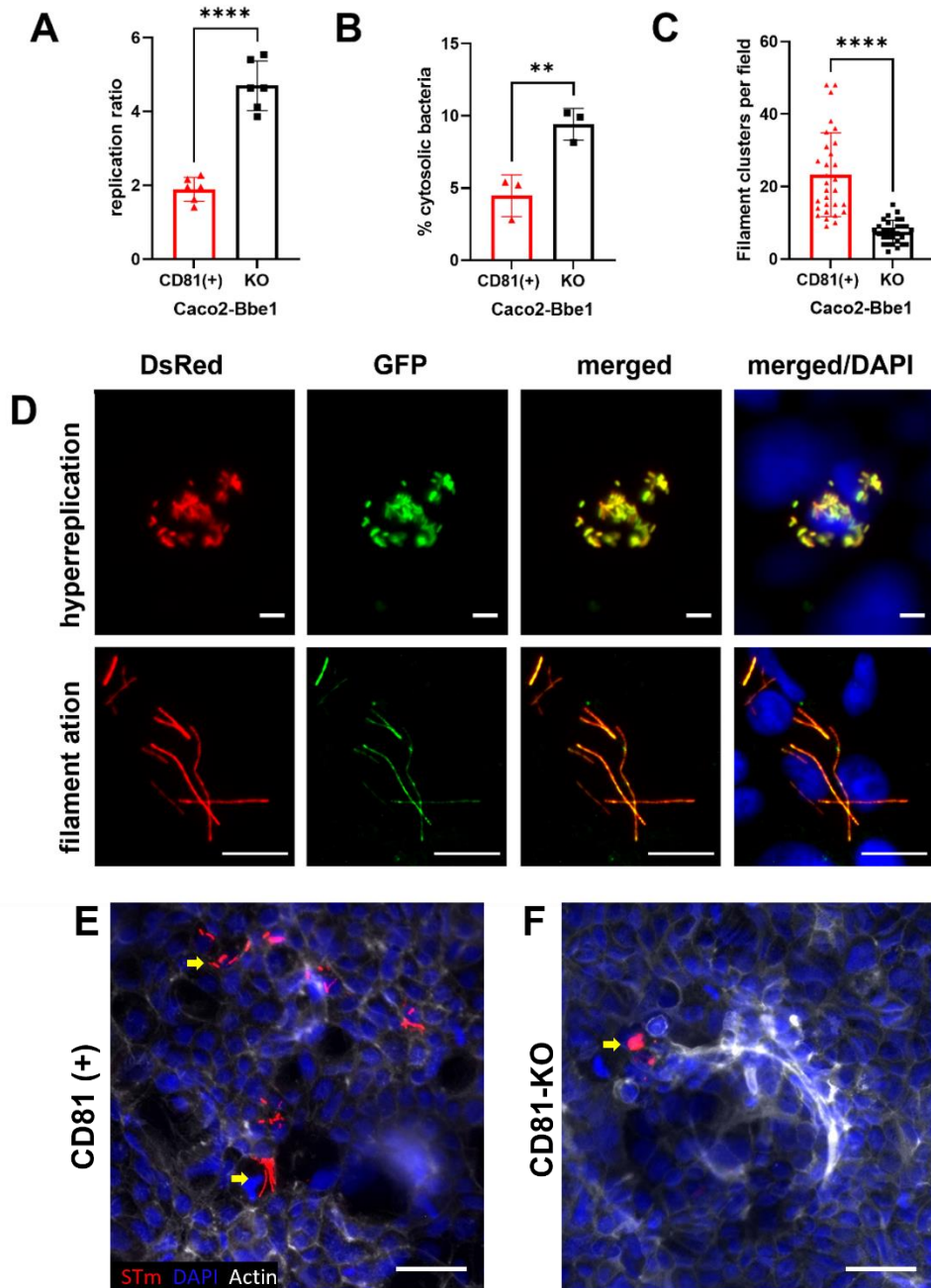


Figure 24. CD81 expression in Caco2-BBe1 influences long-term replication behavior of *Salmonella enterica* serovar Typhimurium SL1344. (A) Knock-out of CD81 influences the replication ratio of *S. Typhimurium* SL1344 in Caco2-BBe1 positively. (B) Chloroquine resistance assay reflects a higher proportion of cytosolic *Salmonella* in Caco2-BBe1 cells not expressing CD81. (C) Incidence of formation of *Salmonella* filamentous growth morphology is higher in CD81-expressing Caco2-BBe1 compared to its knockout counterpart. (D). Occurrence of cytosolic hyperreplicating salmonellae (upper row panels, Scale bar 5µm) and filamentous-growing salmonellae (lower row panels, scale bar 20µm) in Caco2-BBe1 cells using *S. Typhimurium* SL1344 strain expressing DsRed constitutively and and arabinose-inducible GFP promoter. (E) CD81(+) Caco2-BBe1 harboring intracellular filamentous *S. Tm* SL1344 (yellow arrows), 24hpi (Scale bar 50µm), whereas in CD81-deficient Caco2-BBe1 cells (F), *S. Typhimurium* hyperreplicates (yellow arrows) in the cytoplasm, 24hpi (Scale bar 50µm). Data are the representative mean $\pm$ S.D. and were compared using a Student's t-test where **P < 0.01 and ****P < 0.0001.

Discussion

1. *Salmonella* invades host cells in a CD81-dependent process

It is well documented that many pathogens hijack tetraspanins to their own advantage to facilitate colonization of host cells [103], [147] throughout various steps of the infection processes. In recent years, several studies have shown that tetraspanins contribute to the entry and invasion process of various pathogens including viruses[65], [147]–[149], bacteria [93], [94], [111], [150] and parasites[96], [151]. Among the tetraspanins, CD81 is one of the most well-documented tetraspanin reported for its pathologic role in infection and cancer metastasis [69], [103], [152], [153] In addition, its complete crystal structure has been resolved [61].

Our results show that overexpression of human CD81 in HepG2 cells led to an enhancement of *Salmonella* invasion compared to wild-type, CD81-deficient HepG2 cells. Furthermore, we observed this effect to be conserved among representative typhoidal and non-typhoidal *Salmonella* strains, clearly indicating an active involvement of CD81 during *Salmonella* invasion.

Overexpression of human CD81 has been reported to enhance HCV and *Plasmodium* invasion into HepG2 cells [96]–[98]. Additionally, CD81 has been proven to be a receptor for the HCV E2 glycoprotein [98], [101], [102], [125], [126], [154]. Furthermore, in a study by Koutsodakis and colleagues (2007), they found that the level of CD81 cell surface expression in Hepatoma cell lines influences the entry of Hepatitis C virus into host cells [155]. Using the same HepG2 system with CD81 overexpression, Silvie and colleagues in 2006 found that *Plasmodium berghei* sporozoite invasion positively correlates with CD81 overexpression [97]. These studies suggest that CD81 supports entry mechanisms of various pathogens into host cells.

S. Typhimurium (and its monophasic serovar 1,4,[5],12:i), *S. Enteritidis* and *S. Infantis* are the four leading strains of *Salmonella* associated with human salmonellosis most commonly reported in the European Union (EFSA/ECDC report, 2021) [19]. On the other hand, *S. Paratyphi A* is an important emerging typhoidal *Salmonella* strain due to its changing multi-drug resistance pattern and high prevalence in Asia [24], [156]. Given the perceived medical importance of these *Salmonella* strains, infection experiments were carried out using these serovars. Invasion of all these serovars (*S. Typhimurium*, *S. Infantis*, *S. Enteritidis* and *S. Paratyphi A*) were found to be CD81-dependent, suggesting that CD81 is a host factor that could be broadly involved in any of the known invasion pathways of *Salmonella*.

We noted however that in terms of invasiveness towards HepG2, *S. Infantis* 119944 and *S. Enteritidis* LA5 invaded more efficiently than *S. Paratyphi A* 45157 or *S. Typhimurium* 14028s. The genome of *S. Enteritidis* LA5 encodes several complete *Salmonella* pathogenicity islands, and the large plasmid encodes the *spv* genes, which is consistent with the strain's high level of virulence [25]. On the other hand, *S. Infantis* 119944 is an emergent strain originally isolated in Israel harboring a large megaplasmid pESI (~280kb), that confers overall increased virulence to avian and mammalian host cell lines and antimicrobial and environmental stress tolerance [26], [27]. This may explain why such differences in terms of invasiveness exist in our model system, whether any of these virulence factors is/are influenced specifically by CD81 would be an interesting direction to investigate further but is not covered in our study.

We also found a species-specific role of human CD81 for the invasion of *Salmonella* into human epithelial cells. Overexpression of rat CD81 in wild-type CD81-deficient HepG2 cells did not increase the efficiency of *Salmonella* invasion compared to an overexpression of human CD81. We assumed that there is a species-specificity for tetraspanins to cluster together based on their sequence homology as this affects further the protein folding and exposed interaction residues for partner proteins. A study by Flint *et. al.*, 2006 found that overexpression of rat CD81 in HepG2 cells also attenuates HCV infectivity [101]. This may be because human CD81 bears a unique binding site for the HCV E2 ectodomain at the LEL loop, as Homsy and Lang (2017) also demonstrated that

the LEL α -helices are also important for homomeric clustering among CD81 molecules and heteromeric clustering with their partner proteins (*i.e.*, CD9 or CD151)[64]. It could be surmised that a similar mechanism happens during *Salmonella* invasion which could potentially explain the absence of any increase in *Salmonella* invasion (compared to wild-type HepG2) when rat CD81 is overexpressed. To our surprise, overexpression of human CD9 in our HepG2 model further increased *Salmonella* invasion and we further investigated if this is related to human CD81 trafficking towards *Salmonella* entry and invasion site, as discussed later.

Since one of the primary targets for infection of *Salmonella* are intestinal epithelial cells, we utilized Caco2-BBe1 cells to represent an enterocyte infection model and HT29-MTX-E12 cells as the mucus-producing goblet cell-like model. Furthermore, we generated CD81-deficient cells out of these cell lines via CRISPR/Cas9. Infection of these cell lines with *Salmonella* SL1344 showed that the loss of CD81 expression in these cell lines attenuates *Salmonella* invasion, further validating the importance of CD81 on the host cell surface during *Salmonella* invasion.

Earlier studies have found that downregulating CD81 expression in HeLa cells using siRNA reduces *Listeria monocytogenes* invasion [93]. This observation further validates our observation in Caco2-BBe1, where a knockout of CD81 leads to less efficient invasion of *Listeria monocytogenes*. Furthermore, previous studies have also demonstrated that siRNA knockdown of CD81 in primary human hepatocytes reduces the amount of internalized *P. falciparum* exo-erythrocytic forms recovered from infected hepatocytes [151]. On the other hand, other investigators found that a CD81 knockout in A549 cells has no effect on invasion of *Burkholderia pseudomallei* [111]

2. *Salmonella* invasion triggers recruitment of CD81 to the entry site

Using microscopy and image analysis, we observed that CD81 recruitment and redistribution occurs at the invasion foci of *Salmonella* entry site (marked by actin polymerization and membrane ruffle formation) as early as 15 minutes of infection.

Surprisingly, suppressing *Salmonella* uptake by incubating the infected cells at 4°C inhibited trafficking of CD81 into the *Salmonella* adhesion site. These observations suggest that CD81 does not play a role in *Salmonella* adhesion but only during an active invasion event. Since *Salmonella* strains used for infection experiments were cultivated under SPI-1 inducing conditions, coupled with observations of massive membrane ruffle and actin polymerization around the *Salmonella* invasion foci, it could be concluded that CD81 participates in a trigger-like entry of *Salmonella* into host cells. Additionally, we tested a triple mutant S.Tm 14028s/ 3Δ, which lacks the necessary genes for invasion (*invA*, *pagN* and *rck*) and found that they still remain invasive and that CD81 expression improves its efficiency for invasion into HepG2 cells. Furthermore, *rck*-complemented 3Δ mutant, also demonstrate CD81-dependent invasion efficiency into HepG2 cells. Together, these data imply that CD81 supports both trigger- and zipper-like invasion of *Salmonella* into host cells. It has been reported that zipper-like invasion by *Salmonella* can be induced by growing the bacteria in the presence of acyl homoserine lactones and inducing a swarming behavior and expression of SdiA [51], [157]. SdiA, a LuxR homolog which detects AHLs from other bacteria and upregulate the *rck* operon [158], [159]. Furthermore, other investigators have found that *S. Typhimurium* exhibits a discreet invasion mechanism in the murine gut driven by the T3SS-1 effector, SipA [160]. Dissecting further into how exactly is CD81 involved during any of these *Salmonella* invasion mechanisms and which invasins collaborate with CD81 could be an interesting direction to investigate further.

Earlier studies suggest that the role of tetraspanins is related to their ability to interact with other proteins such as adhesion molecule, receptor and co-receptor molecules, major histocompatibility complex antigen, cytoplasmic kinases as well as other tetraspanins [70]. In other studies, tetraspanins have been known to link to the actin cytoskeleton via the proteins ezrin, radixin, and moesin (ERM), and tetraspanin-mediated signaling impacts actin polymerization and remodeling.[83], [129], [161]. In fact, in another study, Coffey *et. al.*, (2009) detected ezrin (an actin-binding protein and a member of ERM family) to colocalize with filamentous actin following CD81 engagement [162]. Furthermore, the intracellular C-terminus tail of CD81, was determined to interact with the small GTPase Rac [83] and several studies have shown that CD81-Rac interaction

regulates cellular motility and migration as well as formation of membrane protrusions in mammalian cells [83], [163], [164]. These previous observations from the side of the host cells might explain the presence of CD81 in membrane ruffles during *Salmonella* invasion, where active actin polymerization and remodeling also occurs in order to internalize the bacteria by macropinocytosis.

Upon stimulation of actin polymerization using epidermal growth factor (EGF), we also noted that CD81 localizes in actin-enriched motility structures. By depleting the level of Rac1-GTP by growing Caco2-BBe1 cells in serum-deficient medium, and inducing Rac1 activation upon addition of EGF, we observed that formation of f-actin in cellular protrusions could be induced as early as 10 minutes. This observation was consistent with the previous findings from a similar experiment performed by Tejera and colleagues (2013) in SUM159 cells [165]. They demonstrated that cellular migration is CD81-dependent and that CD81 interacts directly with Rac1, when they performed cross-correlation analysis of data sets from TIRFM (total internal reflection fluorescence microscopy) and time-lapse images of mCherry-CD81 and GFP-Rac1. Bolshakova and colleagues also demonstrated the same observation of EGF-induced f-actin accumulation in cellular protrusions in A431 cells [130]. Taken together, these previous reports found that CD81 is engaged in actin-enriched cellular structures during cellular stimulation, and further support our observations. It may be surmised that a similar process is triggered during *Salmonella* invasion as several type III secretion effectors such as SipA, SipC, SopE, SopE2 and SptP are also known to regulate actin polymerization directly or indirectly during invasion of host cells [6], [166]. Our results confirm that engagement and localization of CD81 is stimulated by *Salmonella* invasion and perhaps requires a *Salmonella* invasin and is not simply a consequence of *Salmonella* adhesion to host cells. It is not clear which invasins are involved during CD81-mediated internalization of *Salmonella* and this needs further exploration in the future.

3. *Salmonella* invasion can be modulated by targeting CD81.

We were interested in exploring the specificity of CD81-mediated *Salmonella* invasion and to this end, we explored the use of different CD81-specific interactors to perturb CD81 activity. Using anti-CD81 antibody (Clone JS-81) which binds to the LEL region of CD81 [98], we show that *Salmonella* invasion could be reduced at a concentration of 10µg/ml, a concentration lower than this also exerted some reduction in HT29-MTX-E12 cells, but not significantly.

Previously, it has also been shown that CD81 antibodies as well as CD81-LEL peptides could reduce *Salmonella* uptake in macrophages to some extent [107]. We reason that this could be related to the homomeric clustering and oligomerization of CD81 tetraspanin molecules into clusters at the δ -loop (amino acid residues 176- 189) of the LEL based on a series of previous studies [62], [64], [167]. The δ -loop of CD81 is responsible not only for homomeric clustering of CD81 but also for targeting CD9 and CD151 to CD81-containing tetraspanin-enriched microdomain. However, we found that treatment of Caco2-BBe1 with either CD81-LEL prior to *Salmonella* infection did not exert any effect on *Salmonella* invasion compared to control set-ups.

CD81 has been demonstrated as a direct receptor for HCV E2 glycoprotein [101], [126]. Specifically, it has been shown to interact with the LEL domain of CD81 [154], [168], [169]. Krey and colleagues (2010) developed an efficient production system of recombinant HCV E2 ectodomain (E2e), truncated immediately upstream its trans-membrane (TM) region, using *Drosophila melanogaster* cells, and demonstrated that the isolated monomeric E2e binds the soluble CD81 large external loop and efficiently inhibits infection of Huh7.5 cells by infectious HCV particles (HCVcc) in a dose-dependent manner[126]. Additionally, they demonstrated that pull-down assays showed that E2e efficiently binds the CD81 LEL. Similarly, we observed a dose-dependent inhibition of *Salmonella* invasion upon HCV E2e treatment in HepG2 cells overexpressing CD81 but not in the CD81-deficient HepG2 cells. This strengthens further the evidence that *Salmonella* invasion is positively and specifically influenced by surface expression of

CD81, since no effect was seen on wild-type CD81-deficient HepG2 cells when treated with HCV E2e.

Other studies have noted the importance of other CD81 domains outside the LEL region for pathogen invasion [99]. For instance, Banse *et. al.* demonstrated that replacing the human CD81 non-LEL region with that from *Xenopus tropicalis* or *Danio rerio* retains HCV pseudoparticle infectivity while a chimeric construct of human CD81-LEL region with a CD81 backbone of *Caenorhabditis elegans* or *Drosophila melanogaster* abrogates HCVpp infectivity due to critical amino acid substitutions at the TM4 region [99].

Earlier studies have demonstrated the importance of cholesterol in *Salmonella* invasion and its role in the PI3K-dependent anti-inflammatory pathway activation in intestinal epithelial cells. It was shown that depleting cholesterol with methyl- β -cyclodextrin -a water soluble oligosaccharide containing a hydrophobic cavity and has a high capacity to bind cholesterol [123] at the cell surface decreases *Salmonella* internalization in infected Caco2 cells [170], [171]. Cholesterol depletion also reduces *Listeria monocytogenes* invasion into Vero and L2071hEcad cells [120]. Furthermore, It has been shown that cells at the metaphase stage and are abundant in cholesterol at the cell surface are preferential targets for *Salmonella* invasion [122].

In our study, we found that cholesterol depletion prior to *Salmonella* infection of Caco2-BBe1 cells is more pronounced in the presence of CD81 since cholesterol depletion in CD81-KO cells did not further decrease *Salmonella* invasion as much as in CD81-expressing cells after cholesterol depletion. Other investigators have established and characterized the biophysical interactions between cholesterol and various tetraspanins [172]. By using anti-CD81 antibodies that only bind to CD81 associated in a tetraspanin web, it was demonstrated that cholesterol depletion affects the organization and association of CD81 in tetraspanin-enriched microdomains [97]. They demonstrated that cholesterol depletion specifically affects CD81-dependent invasion of malaria sporozoites (utilized by *Plasmodium yoelii* but not by *P. berghei*) into hepatocytes.

Elucidation of the CD81 structure revealed a cholesterol-binding pocket and that cholesterol binding allows CD81 to adopt a closed conformation whereas the lack of it leads into an open conformation [61]. This modulates CD81 activity in cells and suggests a potential mechanism for tetraspanin function regulation. For instance, the B-lymphocyte surface antigen, CD19 is exported efficiently into the cell surface by binding with CD81 when CD81 assumes an open conformation (not bound to cholesterol). Cholesterol binding into CD81 favors a closed conformation, makes the LEL domain inaccessible to CD19 binding, and therefore leads to less efficient export of CD19 [61], [77].

These findings were further expanded by Palor *et. al.* where they demonstrated through molecular dynamic simulations that sequestration of cholesterol out of the CD81 binding pocket, renders the molecule to assume an open conformation and consequently, function poorly as a receptor. Furthermore, mutations at the cholesterol binding pocket modulates HCV entry and alters the tetraspanin's network of molecular partners [119], [173].

4. CD9 localizes at *Salmonella* invasion site together with CD81

We found that overexpression of human CD9 in HepG2 cells also increased *Salmonella* invasion and hypothesized that CD9 also localizes into a tetraspanin-enriched microdomain during *Salmonella* invasion. Indeed, our microscopy data reveal that CD9 is recruited at the *Salmonella* invasion foci upon infection of Caco2-BBe1 cells. Furthermore, we found that CD9 overexpression in HepG2 cells does not affect *Salmonella* adhesion but only *Salmonella* internalization into the host cells leading us to hypothesize that this effect is also connected to an active *Salmonella* invasion.

CD9 is a tetraspanin often reported to be an interactor with CD81[111], [174]–[176]. Following a double immuno-staining of CD81 and CD9, we observed that they co-localize together at the invasion site. We assumed that these tetraspanins are interaction partners that are trafficked together at the *Salmonella* invasion foci. To test this, we employed an

In situ proximity ligation assay (PLA) which is a method that determines the physical closeness of proteins in tissue sections or cell cultures. In this technique, a signal is produced only if the two proteins are closer than 40 nm [145]. Indeed, PLA signals obtained from CD81-CD9 complexes suggest that these tetraspanins could have protein-protein interaction. Taken together, we hypothesize that these tetraspanin partners are contained within a tetraspanin-enriched microdomain which might act to localize certain host receptors together with its cognate bacterial ligands during *Salmonella* invasion. In the future, identifying which other host and bacterial factors are contained within these tetraspanin clusters will be worthy of further investigation.

It was demonstrated that CD9 and CD81 tend to co-precipitate in co-IP experiments along with a partner protein CD9-P1. Using weaker detergents also show that CD63 and CD151 associate within this complex. This suggests that CD81 and CD9 form primary interactions whereas other additional partner proteins are complexed at the tetraspanin web with weaker interactions [174].

Furthermore, other studies have also demonstrated by high-resolution imaging that CD9 recruits ICAM-1 clusters following stimulation with TNF- α stimulation of primary endothelial cells (HUVEC). They noted that these proteins cluster around cellular protrusions, primarily in microvilli and lamellipodia. A knock-down of CD9 by siRNA dysregulates the association of CD9 with adhesion factors JAM-A and ICAM-1 and, consequently reduces cellular protrusions and microvilli formation[177]. They theorized that upon stimulation (*i.e.*, With TNF- α for example) CD9 coordinates microvilli formation (extending to about 100nm from the cell surface) driven by f-actin polymerization and consequent localization of ICAM-1 and JAM-A, which forms a cell surface docking structure (or an adhesion platform).

It could be hypothesized that CD9 clustering (together with other partner host proteins, and CD81) coordinates microvilli formation which serves as docking structure initially, followed by an increasing recruitment of CD81 as membrane ruffling formation becomes more pronounced. Furthermore, CD9 has been demonstrated in several previous studies to be associated with various host factors necessary for cellular adhesion

such as, members of integrin and immunoglobulin superfamily of proteins (as reviewed by Reyes *et. al.* 2018)[178]. Indeed, this is also demonstrated by the studies of Green *et. al.*, and Ventress *et. al.*, where they independently demonstrated that blocking CD9 with antibodies or CD9-LEL peptides decreased the adherence of *Neisseria meningitidis* and *Staphylococcus aureus* in host cell models, respectively. In our experiments, we performed CD9-LEL peptide treatment in Caco2-BBe1 cells, but this did show any effect on *Salmonella* host association or internalization [94], [150].

Two previous studies independently observed that CD81 is coupled to Rac, independent of its activation and this direct interaction is essential for formation of membrane protrusions and cell migration [164], [165]. It could be speculated that tetraspanins CD9 and CD81 could potentially play a role in membrane curvature and protrusion processes considering their high degree of localization in such cellular structures.

Following our microscopy observations, we observed that CD9 clusters around *Salmonella* entry sites but without much pronounced f-actin polymerization (indicating it is recruited to the tetraspanin microdomain prior to the appearance of pronounced membrane ruffling). This was quite different from what we saw from CD81 localization during *Salmonella* invasion, where CD81 localizes around pronounced membrane ruffles driven by cytoskeletal rearrangement. Dissecting the trafficking dynamics of these tetraspanins with techniques that enable higher spatio-temporal resolution, *i.e.*, by live cell imaging or single molecule localization microscopy[179] would provide insights to better understand how *Salmonella* hijacks these tetraspanins during invasion into host cells.

5. Human CD81 impacts intracellular survival of *Salmonella*

We observed that overexpression of either human CD81 and CD9 (but not rat CD81) in HepG2 cells, not only enhance *Salmonella* invasion but also favors *Salmonella* replication during long-term infection. Conversely, knocking out CD81 in HT29-MTX-E12

attenuates *Salmonella* intracellular replication. Together, these observations confirm that human CD81 and human CD9 positively regulates not only *Salmonella* invasion but also further affects the progression of *Salmonella* replication intracellularly.

We found however, that deletion of CD81 in Caco2-BBe1 cells led to a higher replication ratio in CD81-deficient cells. This piqued our interest to investigate further and found that CD81-deficient cells harbor a higher proportion of cytosolic salmonellae (based on chloroquine resistance assay results). CD81-expressing Caco2-BBe1 on the other hand, contain more filamentous non-septated *Salmonella* upon microscopy. These observations might explain the lower CFU counts recovered and consequently the lower replication ratio in CD81(+) Caco2-BBe1 compared to CD81(-) Caco2-BBe1 cells.

Aside from driving formation of cellular protrusions associated with locomotion and pathogen-invasion, CD9 and CD81 were also reported to play a role in membrane fusion. In fact, Sangsri *et. al.*, demonstrated that a deletion of CD81 in A549 cells (human alveolar cells), limited the size and amount of multi-nucleated giant cells formed after a 12-hour infection with *Burkholderia pseudomallei* [111]. An opposite effect was observed by Takeda *et. al.*, where they demonstrated that treatment of monocytes with anti-CD9 and anti-CD81 enhanced membrane fusion and the combination of which, had a cumulative effect, indicating that these tetraspanins may play varying regulatory effects depending on the types and origin of cellular model used, as this may also recruit various tetraspanin partner proteins [144], [180]. Tetraspanins CD81 and CD9 have also been implicated to play a role in egg-sperm fusion as CD9 *-/-* mice are rendered infertile and overexpression of CD81 partially compensates and restores fertility in CD9 *-/-* mice [181], [182].

Upon *Salmonella* invasion, massive membrane ruffling forms around the invasion foci which surrounds and engulfs the bacteria into a host membrane-derived phagosome called a Salmonella-containing vacuole (SCV) in which it replicates [183]. Aside from causing membrane ruffling, the injection of type III secretion effectors also causes formation of host membrane-derived empty macropinosomes which later on fuse with the growing SCV, failure to do so has been demonstrated to cause rupture of the SCV and escape of the bacteria into the cytoplasm. These events were demonstrated by imaging

experiments performed at high temporal and ultrastructural resolution by independent studies[184], [185] .

CD9 and CD81 have been shown to play a role in membrane fusion [110], [111], [182], [186]. It is possible that they also play a role in the maintenance of *Salmonella* in an SCV-bound state either by contributing to membrane flexibility, curvature, and expansion and by fusion with macropinosomes, intraluminal vesicles and/or formation of *Salmonella*-induced filaments (SIFs). Indeed, a review by Zhang and Huang (2012) noted the presence of several tetraspanins in intracellular membrane compartments and inferred that tetraspanins may modify the biophysical characteristics of the cell membrane, such as its curvature, and/or the membrane connections of the cytoskeletal network, to control the morphogenesis of membrane tubular structures[187]. Moreover, a study by Artanavis-Tsakonas *et. al.* (2011) found that another tetraspanin, CD82 is recruited to phagosomes containing internalized bacterial pathogens (*Escherichia coli* and *Staphylococcus aureus*) and fungal pathogens (*Cryptococcus neoformans*, *Candida albicans* and *Aspergillus fumigatus*) and is independent of the recruitment of tetraspanin CD63 [188]. Furthermore, Martinez-Lorenzo *et. al.*, 2001 and Henry *et. al.*, 2005 demonstrated that the mature SCVs are also positive for the presence of tetraspanin CD63 aside from the late endosomal markers LAMP1, vATPase and Rab7 from 8 hours to 48 hours until the bacterial cells are exocytosed [189]. As tetraspanins have been known to exhibit functional redundancy and interact with each other [190], [191], it is possible that the overexpression of CD9 and CD81 in HepG2 in our experiments could facilitate efficient growth of *Salmonella* in an SCV bound state together with CD63 [189]. We speculate that these tetraspanins might help in the organization and proper localization of SPI-2 effectors and host proteins necessary for the maintenance and maturation of the SCV as certain SPI-2 effectors have been demonstrated to drive endosomal tubulation [192]. We have demonstrated that deletion of CD81 in Caco2-BBe1 leads to hyper-replicating salmonellae in the cytosol perhaps caused by premature rupture of CD81-deficient SCV, as the immature SCV is derived largely from the host cell membrane. These observations lead to more exciting directions to explore further because this suggests that CD81 (and CD9 and other tetraspanins) contribute to persistence of *Salmonella* during long-term infection. As these observations are very preliminary, our speculations can be further validated by

investigating the roles of CD81 and CD9 during long term infection with *Salmonella*, from their potential role in maintaining the bacteria in an SCV-bound state, to SCV maturation up to its egress and transcytosis into the extracellular milieu.

6. Theoretical model for the role of CD81 and CD9 in *Salmonella* invasion

Putting all the evidence together, a theoretical framework (Figure 25) for the role of CD81 and CD9 during early *Salmonella* invasion in epithelial cells is proposed. Upon attachment and invasion of *Salmonella* at the entry site, CD81 and CD9 which are both ubiquitously present in the cell surface, are activated and recruited as CD81-CD9 complexes at the site of the invasion foci. Formation of these CD81-CD9 complexes may be due to the action of delivered effectors or the binding of an unknown bacterial ligand to a host receptor and recruitment of other host proteins in a tetraspanin enriched microdomain (TEM). This cascade of events cause membrane ruffling on the host cell surface and localized formation of f-actin polymers at the invasion foci. It is possible that this is triggered through a Rac1-activation dependent process. Previous investigators have noted the clear coupling of CD81 and Rac1 in membrane protrusions during cytoskeletal rearrangements [164], [165], [193], [194] and certain *Salmonella* T3SS-1 effectors and invasins are known to target Rac1 [195]–[197]. This is then followed by macropinocytic uptake and, internalization of the bacteria into the host cell into a *Salmonella* containing vacuole.

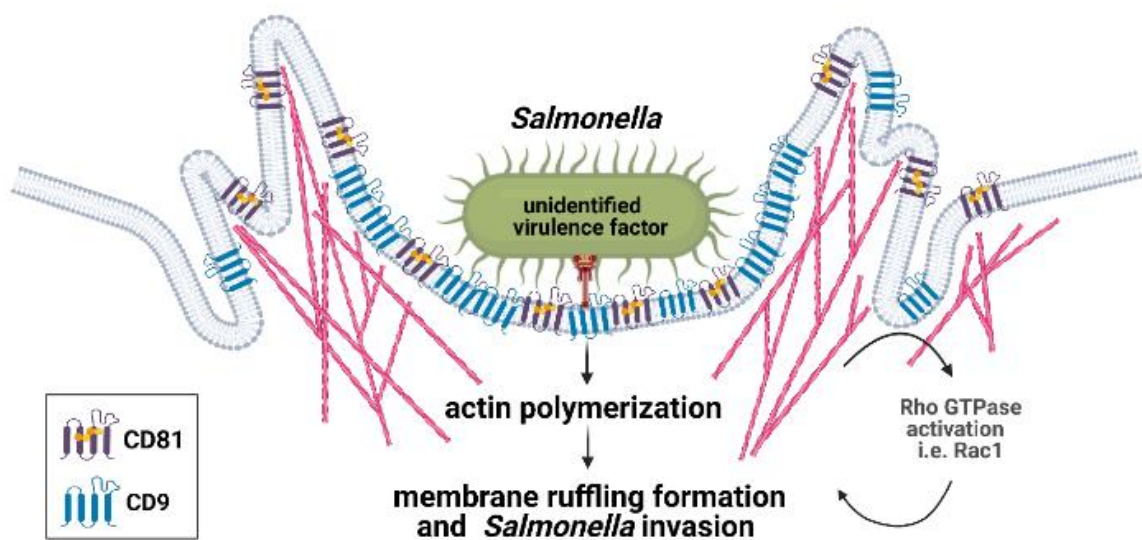


Figure 25. Theoretical framework for the role of CD81 and CD9 during *Salmonella* invasion. CD81 and CD9 is possibly recruited only during *Salmonella* invasion when actin polymerization is triggered by a yet to be identified virulence factors or invasins. CD81 re-localization along actin polymers has been shown to be tightly linked to Rac1 GTPase activation which is most commonly targeted by *Salmonella* Type III secretion effectors during salmonella invasion.

Outlook

In this project, we have established a role of CD81 during *Salmonella* invasion in host cells using various cellular infection models. HepG2, HT29-MTX-E12 and Caco2-BBe1 have been utilized as models to investigate *Salmonella* invasion in a CD81-dependent context. It was interesting to note that CD81 contributes to *Salmonella* invasion to varying degrees, depending on the *Salmonella* strain used. It is possible that these are due to inherent strain variability because of additional virulence factors expressed by other *Salmonella* strains and could be an interesting direction to investigate.

Imaging experiments provided insights into the spatio-temporal dynamics of *Salmonella* invasion and recruitment and localization of host proteins at the invasion foci. With these, we were able to visualize the cooperative role of CD81 and CD9 during *Salmonella* invasion. Additionally, we provided proof that CD81 is recruited only during invasion (coupled with actin polymerization during bacterial internalization) but not at the time of *Salmonella* adhesion on the host cell surface. Time-series experiments or live cell imaging to further resolve the recruitment dynamics of each tetraspanin into a tetraspanin-enriched microdomain might further clarify the exact kinetics of this phenomenon. Furthermore, microscopy experiments can be further extended to more physiologically relevant systems such as in human organoid models to better represent various cell types of the intestinal tract during infection.

Several partner host proteins have been identified by previous studies, using *in silico* modelling, as well as biochemical and microscopic approaches and these could lead us into which subset of host proteins are recruited during *Salmonella* invasion. Additionally, the variability and diversity of protein recruitment and interactions formed at the TEMs pose exciting avenues for exploration in the future. Towards this direction, we are also currently speculating which pathogen and host ligands potentially localize in TEMs during this process.

We found that CD81 does not only play a role during the initial phases of *Salmonella* invasion in host cells but also towards the intracellular lifestyle of *Salmonella*. This provides us some insights that CD81 actively plays a role during long-term infection and during the dynamic maintenance of the *Salmonella*-containing vacuole which could be an exciting prospect to explore further. Additionally, downstream signaling pathways influenced by CD81, could be explored in this direction as well.

Finally, we demonstrated that modulating CD81 activity by targeting different CD81-associated domains or molecular partners, using different biomolecules impacts *Salmonella* invasion. These experiments can be further extended to longer infection timepoints to investigate their effects towards *Salmonella* replication as well as host responses. Furthermore, the use of such agents offer a potential avenue for further exploring tetraspanins as potential drug targets for designing chemotherapeutic agents for microbial infections and other diseases [108], [112], [198].

Materials and Methods

1. Cell culture conditions

HepG2, HEK293T and HT29-MTX-E12 cells were grown in T75 flasks in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Merck) and 1x non-essential amino acids (Gibco). Caco2-Bbe1 cells (kind gift from Prof. Michael Hensel) were maintained in the same medium with the addition of 10 µg/ml holotransferrin (Sigma). Cells were routinely split upon reaching ~80% confluence and were used for infection experiments. All cell lines were maintained in a humidified cell culture incubator at 37 °C with 5% CO₂ and 80% relative humidity.

2. Bacterial inoculum preparation

All *Salmonella* strains used as inocula for infection experiments were prepared from frozen glycerol stocks and were grown in lysogeny broth (LB) with appropriate antibiotics for proper selection (refer to Table 7 for complete list of bacterial strains used) for 16-18 hours at 37°C with shaking at 200 rpm. Bacteria were sub-cultured at a 1:30 dilution in antibiotic-free LB medium for ~3 hours at 37°C until an optical density of 0.5 at 600 nm to obtain *Salmonella* population at an early log phase of growth. *Listeria monocytogenes* EGD was grown from a frozen glycerol stock in 10 ml Brain Heart Infusion (BHI) broth incubated under the same conditions and was sub-cultured at a 1:10 dilution until 0.5 O.D. at 600 nm was reached.

3. Tetraspanin overexpression in HepG2 cells

HepG2 cells were transduced to overexpress CD81r CD9 or rat CD81 as previously described [199] using a lentiviral packaging system approach based on the method previously described by Dull *et.al.* [200] Briefly, lentiviral pseudoparticles encoding either human CD81, rat CD81 or human CD9 were generated by co-transfecting HEK293T cells altogether in a 3-plasmid system: a plasmid encoding vesicular stomatitis virus G (VSV-G) protein, a plasmid encoding HIV gag-pol as well as pTRIP-hCD81, pTRIP-CD9 or pTrip-rCD81 (a kind gift from Charlie Rice and Thomas von Hahn) all mixed in a 1:3:3 ratio, respectively. HepG2 cells were seeded at 8.0×10^4 cells per well of a six-well plate and infected 24 h later with the packaged lentivirus in DMEM supplemented with 3% FBS and 4 µg/ml Polybrene (Sigma Cat. No. TR-1003-G) for 6 hours followed by replenishment with fresh culture medium. Cells were allowed to expand for 48 h post-transduction. Cells were then stained with APC-conjugated monoclonal antibodies (using dilutions

as per manufacturer recommendations) targeting the tetraspanin of interest (refer to Table 4 for a list of antibodies) to analyze its surface expression by flow cytometry. Transduced cells were gated and manually sorted by Fluorescence Activated Cell Sorting using Becton-Dickinson FACS Aria III Fusion under Class-II biosafety conditions, by the Hannover Medical School FACS core facility. Flow cytometry analyses were done by using FlowJo v.10 software.

4. Generation of CD81-deficient intestinal cell lines by CRISPR/Cas9

CD81 synthetic guide RNA (5'-TGGTGGTCTGCGGGTCATGG-3') or a scrambled sequence as control guide RNA (5'-CTAAGGTTAAGTCGCCCTCG-3') cloned into pLentiCRISPR V2 [201] and was incorporated into a lentiviral packaging system along with plasmids encoding VSV-G and HIV-gag/pol and were produced in HEK293T cells as described earlier. Caco2-BBe1 and HT29-MTX E12 cells seeded at 3×10^5 in 6-well plates were transduced with the lentiviral particles encoding either the CD81 guide RNA or scrambled sequence the following day (for at least 4 hours) and replenished with fresh culture medium for further incubation. Selection with 2.5 $\mu\text{g/ml}$ puromycin (HT29-MTX-E12) or 5 $\mu\text{g/ml}$ puromycin (for Caco2-BBe1) was done 48 hours post-transduction and was continued for 3-5 days followed by cell expansion. Cells were stained with anti-human CD81-APC (BD Bioscience 561958) or isotype-matched antibody using dilutions as recommended by the manufacturer. Cells were gated for single cells not expressing CD81 and were sorted through fluorescence activated sorting by negative selection using Becton-Dickinson FACS Aria III Fusion by the Hannover Medical School FACS core facility. CD81(-) single cells were seeded into fresh culture medium, expanded and maintained in medium containing 1 $\mu\text{g/ml}$ puromycin.

5. Reverse transcription and Quantitative PCR

Total RNA was extracted using High Pure RNA Isolation Kit (Roche) and reverse transcription and cDNA synthesis was done using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with manufacturer's instructions. Quantitative real time PCR was performed on a CFX96 Real Time Detection System (Bio-Rad) using the SYBR Green PCR Master Mix (Applied Biosystems). Gene specific primers are Listed in Table 8. and data were normalized to the housekeeping gene for GAPDH following the $\Delta\Delta\text{Ct}$ method for gene expression relative quantification [202].

6. Adhesion assay

To test for the influence of tetraspanins in bacterial adhesion of *Salmonella*, host cells were infected at an MOI of 1:100 and bacterial uptake was inhibited by incubating the cells at 4°C for 60 minutes. Cell lysates were collected, diluted in PBS and plated in LB agar plates and incubated at 37°C overnight. CFUs were counted the next day. %Adhesion was calculated as the ratio of the CFU/well recovered from the cell lysates to that of the CFU/well of inoculum.

7. Gentamicin Killing Assay

HepG2 and HT29-MTX-E12 cells were seeded in 24-well plates at a density of 2×10^5 cells/well one day prior to infection. Caco2-BBe1 cells were seeded at 1.0×10^5 cells in 24-well plates 48 hours prior to infection. Cells were cultured in their respective media and incubated in 5% CO₂ at 37°C. One hour prior to infection, cells were resuspended in serum-free culture medium. Early log-phase *S. Typhimurium* SL1344 or *L. monocytogenes* EGD were harvested and adjusted to a multiplicity of infection (MOI) of 10 in the host cell culture medium. Cells were infected for 30 mins (*S. Typhimurium* SL1344, *S. Enteritidis* LA5, *S. Infantis* 119944 or *S. Paratyphi* A 45157) or 60 minutes (*S. Typhimurium* 14028s, 14028s3Δ or 14028s/3 Δ+rck) at 37°C with 5% CO₂; non-adherent bacteria were removed by washing with Phosphate.buffered Saline solution (PBS) with Mg²⁺ and Ca²⁺ (Gibco).

To enumerate host-associated bacteria, cells were lysed with PBS containing 1% (v/v) Triton X-100 and 0.1% (v/v) sodium dodecyl sulfate (SDS). Cell lysates were collected, diluted in PBS and plated in LB agar plates and incubated at 37°C overnight. CFUs were counted the next day. %host association is calculated as the CFU/well recovered from the cell lysates normalized to that of the CFU/well of inoculum.

For gentamicin killing assay, unbound extracellular bacteria were washed away with PBS after infection and fresh culture medium containing 100 µg/ml gentamicin was added and infected cells were further incubated for one hour to kill remaining extracellular bacteria. After three washes with PBS (with Mg²⁺ and Ca²⁺) internalized bacteria were recovered by washing and lysing the host cells as mentioned earlier. Cell lysates were serially diluted in PBS and plated in LB agar plates (for all *Salmonella* strains) or BHI agar plates (*L. monocytogenes* EGD) and incubated at 37 °C overnight. Bacterial colonies were counted and % invasion was calculated as a ratio of the CFU/well recovered from the cell lysates to that of the inoculum/well.

To enumerate intracellular bacteria at 24hpi, 100µg/ml gentamicin from 1.5hpi were reduced to 10µg/ml and the cells were incubated further up to 24 hours, prior to plating and enumerate of CFUs as mentioned. Replication ratio is calculated as the ratio obtained from CFU/well of infected tissue cultures at 24hpi to that of 1.5hpi.

8. Chloroquine resistance Assay

Cells were seeded as mentioned previously in preparation for infection and gentamicin killing assay was performed. At 1.5hpi, growth medium containing 100µg/ml gentamicin concentration was reduced to 10µg/ml and infected cells were incubated further overnight. At 23 hpi, growth medium was exchanged with medium containing 10µg/ml gentamicin and 800 µg/ml chloroquine (Sigma, Cat. No. C6628) and incubated further for 1 hr. Cells washed and lysed as mentioned previously and salmonellae were enumerated from cell lysates in the same manner as in gentamicin killing assay. %Cytosolic CFU was calculated as the ratio of the CFU counts recovered from chloroquine killing assay at 24hpi to that of the gentamicin killing assay at 24hpi.

9. Antibody or LEL-derived peptide blocking

Truncated regions of CD81 or CD9 bearing the LEL region were produced as recombinant proteins according to Krey *et. al.*[203] and were termed “CD81-LEL” and “CD9-LEL” peptides (kind gift from Prof. Thomas Krey). These were diluted to a final concentration of 20µg/ml in cell culture medium. For a complete list of antibodies, please refer to Table 2. Anti-CD81 (JS-81) or the isotype-matched antibody were of the IgG1 subclass were diluted to a concentration of 10µg/ml, 5µg/ml and 2.5µg/ml in cell culture medium. Cells were pretreated with recombinant LEL peptides or monoclonal antibodies and matched controls for 1 hour at 37°C prior to infection experiment.

10. HCV E2 blocking

Recombinant HCV E2 was produced as described (Krey *et. al.*, 2010). Binding of soluble E2 to HepG2 cells expressing human CD81 was as previously described (Zhang *et. al.*, 2004). Briefly, 2×10^5 HepG2 cells were incubated with soluble HCV E2 in increasing concentrations from 0.1 µM, 1 µM and 2 µM in serum free media for one hour followed by infection with *S. Typhimurium* SL1344 at MOI 10 for 30 minutes. Gentamicin protection assay was performed

afterwards, and the amount of intracellular *Salmonella* 1.5 h post-infection was enumerated as described above.

11. Cell surface cholesterol depletion

Cell surface cholesterol depletion was carried out according to the methods by [151], [172]. Cell monolayers were incubated with 10mM methyl- β -cyclodextrin (MBCD, Sigma) in serum free medium or serum-free medium only at 37 C for 15 minutes. This was followed by replenishment of normal growth medium with bacterial inoculum. Gentamicin killing assay was then performed as described.

12. Cellular Stimulation with Epidermal Growth Factor

Caco2-BBe1 cells were seeded in coverslips as mentioned previously. Prior to EGF stimulation, cells were grown in serum deficient growth medium for 16 hours. The medium was then replaced with either the same medium or with addition of 100ng/ml of murine EGF (Peprotech Cat. No. 315-09). Cells were stimulated for 10 minutes followed by 3x PBS wash and fixation with 4%PFA for 10 minutes. Cells were stained for F-actin, host nuclei and CD81 as mentioned previously and were examined for immunofluorescence microscopy.

13. Immunofluorescence microscopy

Caco2-BBe1 cells were seeded on coverslips in 24 well plates. After infection, samples were fixed using 4% paraformaldehyde (PFA) for 10 min, washed three times with 1x PBS and blocked and permeabilized with a buffer solution containing 10% normal goat serum, 0.1% Triton X-100, 0.05% Tween 20 in 1x PBS. Bacteria expressing fluorescent proteins were used as reporter strains (Table 7).

Primary and secondary antibodies were diluted in the same buffer (if permeabilization is not needed, Triton X-100 is omitted) as per manufacturer recommendations. A summary of all the list of primary and matching secondary antibodies used for immunofluorescence microscopy are listed in Table 2 and Table 3. Host cell nuclei were detected using 4',6-diamidino-2-phenylindole, dilactate (DAPI, Invitrogen, Cat. No. D3571). F-actin was detected using Phalloidin-iFluor 647 (Abcam, Cat. No. ab176759) and cells were mounted and using fluorescent mounting medium (Dako Cat. No. S3023).

14. Proximity Ligation Assay

Proximity ligation assays were performed upon infection of Caco2-BBe1 cells with GFP-expressing S.Tm SL1344 using the Red Duolink In Situ PLA Kit (Sigma-Aldrich). Briefly, Caco2-Bbe1 cells were seeded in an 8-well chamber slide at 5×10^4 cells per well and were infected with S. Typhimurium SL1344/pFPV25.1.-GFP at an MOI 100 bacteria/host cell. 15 minutes post-infection, cells were washed with 1x PBS three times followed by blocking, PFA-fixation and immunolabelling with anti-CD81 (Invitrogen, Cat. No. PA5-82288) and anti-CD9 (HI9a-RUO clone; Biolegend Cat. No. 312102) as described earlier (refer also to Table 2). Proximity ligation assay was performed using the supplied PLA-probes followed by ligation, amplification and detection reagents as specified in the manufacturer's 'custom solutions' protocol of the DuoLink *In Situ* PLA Kit. Image acquisition was done using a Zeiss Apotome 2 microscope for widefield imaging. Image data were visualized and analysed using Zen Blue 3.4 software.

15. Image Acquisition and Analysis

Microscopy and Image acquisition was done using a Zeiss Apotome 2 or a Leica Thunder Live Cell imaging platform for widefield imaging or with a Zeiss LSM 980 with Airyscan 2 for laser confocal imaging performed at the Research Core Unit for Laser Microscopy, Hannover Medical School. Image data were visualized and analysed using Zen Blue 3.4 or FIJI/ImageJ software [204].

16. Statistical Analysis

Data were analyzed using GraphPad Prism ver. 8.4.3 software (Graphpad Software, LLC). Graphs display the mean values $\pm$ SD and represent three independent biological repetitions unless otherwise stated. One-way analysis of variance (ANOVA) with post-hoc Tukey's multiple comparison test, or unpaired two-tailed t-test, were used in the statistical analysis, as indicated. P-values below 0.05 were considered statistically significant and were presented as ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ or **** $P < 0.0001$.

17. Supplementary list of materials

Table 2. Primary Antibodies used for immunocytochemistry

Target/ Reactivity	Clone	Antibody type	Host	Brand	Catalog no.	RRID no.
Human CD81	JS-81 RUO	Monoclonal	Mouse	BD Bioscience	555675	AB_396028
IgG1 isotype control	MOPC-21	Monoclonal	mouse	BD Bioscience	555746	AB_396088
Human CD81	NA	Polyclonal	Rabbit IgG	Invitrogen	PA5-82288	AB_2789447
Human CD9	HI9a-RUO	Monoclonal	mouse	Biolegend	312102	AB_314907

Table 3. Secondary Antibodies used for immunocytochemistry

Target/Reactivity	Host	Antibody form	Fluorophore	Brand	Catalog no.	RRID no.
anti-rabbit	Goat/ IgG (H+L)	F(ab') ₂ fragment, polyclonal	Alexa Fluor®546	Invitrogen	A-11071	AB_2534115
anti-mouse	Goat/ IgG(H+L)	Polyclonal	AlexaFluor 633	Invitrogen	A-21050	AB_2535718
Anti-mouse	Goat/ IgG (H+L)	Polyclonal	AlexaFlour 546	Invitrogen	A-11003	AB_2534071
Anti-mouse	Goat/IgG (H+L)	F(ab') ₂ Fragment, Polyclonal	AlexaFlour633	Invitrogen	A21053	AB_2535720
Anti-rabbit	Goat/ IgG (H+L)	Polyclonal	AlexaFlour633	Invitrogen	A21072	AB_2535733

Table 4. Conjugated antibodies used for flow cytometry and fluorescence-activated cell sorting

Target	Clone	Antibody type	Host	Fluorophore	Excitation λ (nm)	Brand	Catalog no.	RRID no.
Human CD81	JS-81 RUO	monoclonal	mouse	APC	Red 627-640 nm	BD Bioscience	551112	AB_398491
IgG1-Kappa	MOPC-21	monoclonal	mouse	APC	Red 627-640 nm	BD Bioscience	555751	AB_398467
Human CD81	5A6	Monoclonal	mouse	APC	Red 627-640 nm	Biolegend	349509	AB_2564020
Human CD9	HI9a-RUO	Monoclonal	mouse	APC	Red 627-640 nm	Biolegend	312107	AB_2721450
IgG1-Kappa Isotype Control	MOPC-21	Monoclonal	mouse	APC	Red 627-640 nm	Biolegend	400121	AB_2665396
Rat/mouse CD81	Eat-2	Monoclonal	Armenian hamster	APC	Red 627-640 nm	Biolegend	104909	AB_2562993
IgG1-Kappa Isotype Control	HTK888	Monoclonal	Armenian hamster	APC	Red 627-640 nm	Biolegend	400911	NA

Table 5. Plasmids used for CRISPR/Cas9 deletion of human CD81

Plasmid name	Promoter	Cloning restriction site	Gene insert/ description	Cloning host	Source/reference
pLentiCRISPR V2-CD81	CMV	Multiple cloning sites	Generated by golden gate cloning: pLenti crispr v2 ccdB and annealed forward and reverse oligos encoding for guide RNA (gRNA) were cut with BsmBI. Ligation with T4 ligase. One clone was taken and sequenced	<i>Escherichia coli</i> Turbo NEB	Generous gift from Dr. Gisa Gerold/ Dr. Lisa Lasswitz (Twincore, Hannover, DE)
pczVSV-Gwt	CMV	Multiple cloning sites	VSV-G expression, packaging plasmid for lentiviral particle generation on pcDNA3.1 cloning template	<i>Escherichia coli</i> Turbo NEB	Generous gift from Dr. Gisa Gerold/ Dr. Lisa Lasswitz (Twincore, Hannover, DE)
pCMV-dR8.74 GK	CMV	Multiple cloning sites	HIV-gag, pol, tat, rev expression; packaging plasmid for lentiviral particle generation on	<i>Escherichia coli</i> Turbo NEB	Generous gift from Dr. Gisa Gerold/ Dr. Lisa Lasswitz (Twincore, Hannover, DE)

Table 6. Plasmids used for CD81 overexpression in HepG2

Plasmid name	Promoter	Cloning restriction site	Gene insert/ description	Cloning host	Source/reference
pTRIP-hSEL-hLEL CD81 (human CD81)	CMV	Multiple cloning sites	Refer to Flint <i>et. al.</i> , 2006	<i>Escherichia coli</i> Turbo NEB	Generous gift from Dr. Gisa Gerold/ Dr. Francisco Zapatero-Belinchon
pTRIP-rSEL-rLEL CD81 (rat CD81)	CMV	MCS	Refer to Flint <i>et. al.</i> , 2006	<i>Escherichia coli</i> Turbo NEB	Generous gift from Dr. Gisa Gerold/ Dr. Francisco Zapatero-Belinchon
pTRIP- human CD9	CMV	MCS	Refer to Flint <i>et. al.</i> , 2006	<i>Escherichia coli</i> Turbo NEB	Generous gift from Dr. Gisa Gerold/ Dr. Francisco Zapatero-Belinchon
pczVSV-Gwt	CMV	MCS	Same details as Table 5	<i>Escherichia coli</i> Turbo NEB	Generous gift from Dr. Gisa Gerold/ Dr. Francisco Zapatero-Belinchon
pCMV-dR8.74 GK	CMV	MCS	Same details as Table 5	<i>Escherichia coli</i> Turbo NEB	Generous gift from Dr. Gisa Gerold/ Dr. Francisco Zapatero-Belinchon

Table 7. Bacterial strains used in this study

Species	Serovar	Strain	Genotype	Plasmid	Resistance
<i>Salmonella enterica</i>	Typhimurium	SL1344	wild-type		Streptomycin (100µg/ml)
<i>Salmonella enterica</i>	Typhimurium	14028s (NCTC 12023)	wild-type		None
<i>Salmonella enterica</i>	Enteritidis	LA5	wild-type		Nalidixic Acid (20µg/ml)
<i>Salmonella enterica</i>	Infantis	119944	wild-type		tetracyclin (20µg/ml)
<i>Salmonella enterica</i>	Paratyphi A	45157	wild-type		no
<i>Listeria monocytogenes</i>	EGD	Serotype 1/2a	wild-type		no
Reporter strains for microscopy					
<i>Salmonella enterica</i>	Typhimurium	SL1344	GFP+	pFPV25.1-GFP	Ampicillin (100µg/ml)
<i>Salmonella enterica</i>	Typhimurium	SL1344	EM7::DsRed; PuhpT::sfGFP	p4889 dsRed/sfGFP	Amp / Carbenicillin 50 µg/ml
			dsRed constitutive GFP inducible in cytosol		

Table 8. PCR Primers used in this study

Gene target/Name	Directionality	Sequence (5' to 3')
CD9	Forward	TCGCCATTGAAATAGCTGCGGC
CD9	Reverse	CGCATAGTGGATGGCTTTCAGC
Human GAPDH	Forward	GGCATCCTGGGCTACACTGA
Human GAPDH	Reverse	GGAGTGGGTGTCGCTGTTG

Literature Cited

- [1] A. Beceiro, M. Tomás, and G. Bou, "Antimicrobial Resistance and Virulence: a Successful or Deleterious Association in the Bacterial World?," *Clin Microbiol Rev*, vol. 26, no. 2, pp. 185–230, Apr. 2013, doi: 10.1128/CMR.00059-12.
- [2] J. E. Galán and H. Wolf-Watz, "Protein delivery into eukaryotic cells by type III secretion machines," *Nature*, vol. 444, no. 7119, pp. 567–573, Nov. 2006, doi: 10.1038/nature05272.
- [3] M. E. J. Woolhouse, J. P. Webster, E. Domingo, B. Charlesworth, and B. R. Levin, "Biological and biomedical implications of the co-evolution of pathogens and their hosts," *Nat Genet*, vol. 32, no. 4, pp. 569–577, Dec. 2002, doi: 10.1038/NG1202-569.
- [4] T. V. DOUGNON *et al.*, "Pathogenicity, Epidemiology and Virulence Factors of Salmonella species: A Review," *Not Sci Biol*, vol. 9, no. 4, pp. 460–466, Dec. 2017, doi: 10.15835/nsb9410125.
- [5] O. Gal-Mor, E. C. Boyle, and G. A. Grassl, "Same species, different diseases: how and why typhoidal and non-typhoidal Salmonella enterica serovars differ," *Front Microbiol*, vol. 5, Aug. 2014, doi: 10.3389/fmicb.2014.00391.
- [6] A. Haraga, M. B. Ohlson, and S. I. Miller, "Salmonellae interplay with host cells," *Nat Rev Microbiol*, vol. 6, no. 1, pp. 53–66, Jan. 2008, doi: 10.1038/nrmicro1788.
- [7] S. Ménard, S. Lacroix-Lamandé, K. Ehrhardt, J. Yan, G. A. Grassl, and A. Wiedemann, "Cross-Talk Between the Intestinal Epithelium and Salmonella Typhimurium," *Front Microbiol*, vol. 13, Jun. 2022, doi: 10.3389/fmicb.2022.906238.
- [8] A. Zadernowska and W. Chajęcka-Wierzchowska, "Rapid, Alternative Methods for Salmonella Detection in Food," in *Foodborne Pathogens and Antibiotic Resistance*, Hoboken, NJ, USA: John Wiley & Sons, Inc., 2017, pp. 203–210. doi: 10.1002/9781119139188.ch8.
- [9] R. G. Ferrari, D. K. A. Rosario, A. Cunha-Neto, S. B. Mano, E. E. S. Figueiredo, and C. A. Conte-Junior, "Worldwide Epidemiology of Salmonella Serovars in Animal-Based Foods: a Meta-analysis," *Appl Environ Microbiol*, vol. 85, no. 14, Jul. 2019, doi: 10.1128/AEM.00591-19.
- [10] K. D. MacKenzie, M. B. Palmer, W. L. Köster, and A. P. White, "Examining the Link between Biofilm Formation and the Ability of Pathogenic Salmonella Strains to Colonize Multiple Host Species," *Front Vet Sci*, vol. 4, Aug. 2017, doi: 10.3389/fvets.2017.00138.
- [11] F. W. Brenner, R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan, "Salmonella Nomenclature," *J Clin Microbiol*, vol. 38, no. 7, pp. 2465–2467, Jul. 2000, doi: 10.1128/JCM.38.7.2465-2467.2000.
- [12] S. Issenhuth-Jeanjean *et al.*, "Supplement 2008–2010 (no. 48) to the White–Kauffmann–Le Minor scheme," *Res Microbiol*, vol. 165, no. 7, pp. 526–530, Sep. 2014, doi: 10.1016/J.RESMIC.2014.07.004.

- [13] M. B. Goldberg and R. H. Rubin, "The spectrum of Salmonella infection.," *Infect Dis Clin North Am*, vol. 2, no. 3, pp. 571–98, Sep. 1988.
- [14] J. D. Stanaway *et al.*, "The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017," *Lancet Infect Dis*, vol. 19, no. 4, pp. 369–381, Apr. 2019, doi: 10.1016/S1473-3099(18)30685-6.
- [15] B. W. Brunelle, B. L. Bearson, and H. K. Allen, "Prevalence, Evolution, and Dissemination of Antibiotic Resistance in *Salmonella*," in *Foodborne Pathogens and Antibiotic Resistance*, Hoboken, NJ, USA: John Wiley & Sons, Inc., 2017, pp. 331–348. doi: 10.1002/9781119139188.ch14.
- [16] S. E. Majowicz *et al.*, "The Global Burden of Nontyphoidal *Salmonella* Gastroenteritis," *Clinical Infectious Diseases*, vol. 50, no. 6, pp. 882–889, Mar. 2010, doi: 10.1086/650733.
- [17] J. D. Stanaway *et al.*, "The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017," *Lancet Infect Dis*, vol. 19, no. 12, pp. 1312–1324, Dec. 2019, doi: 10.1016/S1473-3099(19)30418-9.
- [18] "The European Union One Health 2019 Zoonoses Report," *EFSA Journal*, vol. 19, no. 2, Feb. 2021, doi: 10.2903/j.efsa.2021.6406.
- [19] "The European Union One Health 2020 Zoonoses Report," *EFSA Journal*, vol. 19, no. 12, Dec. 2021, doi: 10.2903/j.efsa.2021.6971.
- [20] "The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016," *EFSA Journal*, vol. 15, no. 12, Dec. 2017, doi: 10.2903/j.efsa.2017.5077.
- [21] S. P. Luby, "Bacteria: Salmonella Typhi and Salmonella Paratyphi," in *Encyclopedia of Food Safety*, Elsevier, 2014, pp. 515–522. doi: 10.1016/B978-0-12-378612-8.00113-X.
- [22] WHO, "WHO estimates of the global burden of foodborne diseases: executive summary," 2015. Accessed: Nov. 08, 2022. [Online]. Available: www.who.int
- [23] S. Verma, S. Thakur, A. Kanga, G. Singh, and P. Gupta, "Emerging Salmonella Paratyphi A enteric fever and changing trends in antimicrobial resistance pattern of salmonella in Shimla," *Indian J Med Microbiol*, vol. 28, no. 1, pp. 51–53, Jan. 2010, doi: 10.4103/0255-0857.58730.
- [24] R. L. Ochiai *et al.*, "*Salmonella* Paratyphi A Rates, Asia," *Emerg Infect Dis*, vol. 11, no. 11, pp. 1764–1766, Nov. 2005, doi: 10.3201/eid1111.050168.
- [25] O. Grépinet *et al.*, "Genome Sequence of the Invasive *Salmonella enterica* subsp. *enterica* Serotype Enteritidis Strain LA5," *J Bacteriol*, vol. 194, no. 9, pp. 2387–2388, May 2012, doi: 10.1128/JB.00256-12.
- [26] G. Aviv *et al.*, "A unique megaplasmid contributes to stress tolerance and pathogenicity of an emergent *S almonella enterica* serovar Infantis strain," *Environ Microbiol*, vol. 16, no. 4, pp. 977–994, Apr. 2014, doi: 10.1111/1462-2920.12351.

- [27] G. Aviv *et al.*, "Differences in the expression of SPI-1 genes pathogenicity and epidemiology between the emerging *Salmonella enterica* serovar Infantis and the model *Salmonella enterica* serovar Typhimurium," *J Infect Dis*, vol. 220, no. 6, pp. 1071–1081, Aug. 2019, doi: 10.1093/infdis/jiz235.
- [28] "USDA ERS - Cost Estimates of Foodborne Illnesses." Accessed: Nov. 08, 2022. [Online]. Available: <https://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses.aspx>
- [29] M. E. Ohl and S. I. Miller, "Salmonella: A Model for Bacterial Pathogenesis," *Annu Rev Med*, vol. 52, no. 1, pp. 259–274, Feb. 2001, doi: 10.1146/annurev.med.52.1.259.
- [30] A. Lahiri, A. Lahiri, N. Iyer, P. Das, and D. Chakravorty, "Visiting the cell biology of *Salmonella* infection," *Microbes Infect*, vol. 12, no. 11, pp. 809–818, Oct. 2010, doi: 10.1016/j.micinf.2010.05.010.
- [31] P. Malik-Kale, C. E. Jolly, S. Lathrop, S. Winfree, C. Luterbach, and O. Steele-Mortimer, "Salmonella- at home in the host cell," *Front Microbiol*, vol. 2, no. JUNE, 2011, doi: 10.3389/fmicb.2011.00125.
- [32] D. Hanes, *International Handbook of Foodborne Pathogens- Nontyphoidal Salmonella*. CRC Press, 2003. doi: 10.1201/9780203912065.
- [33] A. Takeuchi, "Electron microscope studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*.,", *Am J Pathol*, vol. 50, no. 1, pp. 109–36, Jan. 1967.
- [34] T. H. Kent, S. B. Formal, and E. H. Labrec, "Acute enteritis due to *Salmonella typhimurium* in opium-treated guinea pigs.,", *Arch Pathol (Chic)*, vol. 81, no. 6, pp. 501–8, Jun. 1966.
- [35] H. W. Florey, "Observations on the functions of mucus and the early stages of bacterial invasion of the intestinal mucosa," *J Pathol Bacteriol*, vol. 37, no. 2, pp. 283–289, 1933, doi: 10.1002/path.1700370210.
- [36] J. E. Galán and R. Curtiss, "Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells.,", *Proceedings of the National Academy of Sciences*, vol. 86, no. 16, pp. 6383–6387, Aug. 1989, doi: 10.1073/pnas.86.16.6383.
- [37] S. L. Marcus, J. H. Brumell, C. G. Pfeifer, and B. B. Finlay, "Salmonella pathogenicity islands: big virulence in small packages," *Microbes Infect*, vol. 2, no. 2, pp. 145–156, Feb. 2000, doi: 10.1016/S1286-4579(00)00273-2.
- [38] C. J. Blondel, J. C. Jiménez, I. Contreras, and C. A. Santiviago, "Comparative genomic analysis uncovers 3 novel loci encoding type six secretion systems differentially distributed in *Salmonella* serotypes.,", *BMC Genomics*, vol. 10, p. 354, Aug. 2009, doi: 10.1186/1471-2164-10-354.
- [39] B. Ilyas, C. N. Tsai, and B. K. Coombes, "Evolution of *Salmonella*-Host Cell Interactions through a Dynamic Bacterial Genome," *Front Cell Infect Microbiol*, vol. 7, Sep. 2017, doi: 10.3389/fcimb.2017.00428.

- [40] P. Velge *et al.*, "Multiplicity of Salmonella entry mechanisms, a new paradigm for Salmonella pathogenesis," *Microbiologyopen*, vol. 1, no. 3, pp. 243–258, 2012, doi: 10.1002/mbo3.28.
- [41] C. M. Collazo and J. E. Galán, "The invasion-associated type-III protein secretion system in Salmonella – a review," *Gene*, vol. 192, no. 1, pp. 51–59, Jun. 1997, doi: 10.1016/S0378-1119(96)00825-6.
- [42] E. Cascales, "Inside the Chamber of Secrets of the Type III Secretion System," *Cell*, vol. 168, no. 6. Cell Press, pp. 949–951, Mar. 09, 2017. doi: 10.1016/j.cell.2017.02.028.
- [43] Z. Boumart, P. Velge, and A. Wiedemann, "Multiple invasion mechanisms and different intracellular Behaviors: a new vision of *Salmonella* -host cell interaction," *FEMS Microbiol Lett*, vol. 361, no. 1, pp. 1–7, Dec. 2014, doi: 10.1111/1574-6968.12614.
- [44] F. Ramos-Morales, " Impact of Salmonella enterica Type III Secretion System Effectors on the Eukaryotic Host Cell ," *ISRN Cell Biol*, vol. 2012, pp. 1–36, Dec. 2012, doi: 10.5402/2012/787934.
- [45] E. J. Heffernan, S. Reed, J. Hackett, J. Fierer, C. Roudier, and D. Guiney, "Mechanism of resistance to complement-mediated killing of bacteria encoded by the Salmonella typhimurium virulence plasmid gene rck.," *Journal of Clinical Investigation*, vol. 90, no. 3, pp. 953–964, Sep. 1992, doi: 10.1172/JCI115972.
- [46] D. M. Cirillo, E. J. Heffernan, L. Wu, J. Harwood, J. Fierer, and D. G. Guiney, "Identification of a domain in Rck, a product of the Salmonella typhimurium virulence plasmid, required for both serum resistance and cell invasion," *Infect Immun*, vol. 64, no. 6, pp. 2019–2023, Jun. 1996, doi: 10.1128/iai.64.6.2019-2023.1996.
- [47] J. Mambu, I. Virlogeux-Payant, S. Holbert, O. Grépinet, P. Velge, and A. Wiedemann, "An updated view on the Rck invasin of Salmonella: Still much to discover," *Frontiers in Cellular and Infection Microbiology*, vol. 7, no. DEC. Frontiers Media S.A., Dec. 08, 2017. doi: 10.3389/fcimb.2017.00500.
- [48] A. M. Crago and V. Koronakis, "Binding of extracellular matrix laminin to *Escherichia coli* expressing the *Salmonella* outer membrane proteins Rck and PagC," *FEMS Microbiol Lett*, vol. 176, no. 2, pp. 495–501, Jul. 1999, doi: 10.1111/j.1574-6968.1999.tb13703.x.
- [49] S. I. Miller, W. S. Pulkkinen, M. E. Selsted, and J. J. Mekalanos, "Characterization of defensin resistance phenotypes associated with mutations in the phoP virulence regulon of Salmonella typhimurium.," *Infect Immun*, vol. 58, no. 11, pp. 3706–10, Nov. 1990, doi: 10.1128/iai.58.11.3706-3710.1990.
- [50] I. Behlau and S. I. Miller, "A PhoP-repressed gene promotes Salmonella typhimurium invasion of epithelial cells.," *J Bacteriol*, vol. 175, no. 14, pp. 4475–84, Jul. 1993, doi: 10.1128/jb.175.14.4475-4484.1993.
- [51] M. Rosselin *et al.*, "Rck of Salmonella enterica, subspecies enterica serovar enteritidis, mediates zipper-like internalization," *Cell Res*, vol. 20, no. 6, pp. 647–664, Jun. 2010, doi: 10.1038/CR.2010.45.

- [52] A. Wiedemann *et al.*, "Identification of the epidermal growth factor receptor as the receptor for Salmonella Rck-dependent invasion.," *FASEB J*, vol. 30, no. 12, pp. 4180–4191, 2016, doi: 10.1096/fj.201600701R.
- [53] A. L. Radtke, J. W. Wilson, S. Sarker, and C. A. Nickerson, "Analysis of interactions of Salmonella type three secretion mutants with 3-D intestinal epithelial cells.," *PLoS One*, vol. 5, no. 12, p. e15750, Dec. 2010, doi: 10.1371/journal.pone.0015750.
- [54] A. Aiastui, M. G. Pucciarelli, and F. García-del Portillo, "Salmonella enterica serovar typhimurium invades fibroblasts by multiple routes differing from the entry into epithelial cells.," *Infect Immun*, vol. 78, no. 6, pp. 2700–13, Jun. 2010, doi: 10.1128/IAI.01389-09.
- [55] S. M. Roche *et al.*, "Salmonella Typhimurium invalidated for the three currently known invasion factors keeps its ability to invade several cell models," *Front Cell Infect Microbiol*, vol. 8, no. AUG, p. 273, Aug. 2018, doi: 10.3389/FCIMB.2018.00273/BIBTEX.
- [56] Q. Hu *et al.*, "Salmonella enterica serovar Senftenberg human clinical isolates lacking SPI-1.," *J Clin Microbiol*, vol. 46, no. 4, pp. 1330–6, Apr. 2008, doi: 10.1128/JCM.01255-07.
- [57] M. A. Lambert and S. G. Smith, "The PagN protein of Salmonella enterica serovar Typhimurium is an adhesin and invasin," *BMC Microbiol*, vol. 8, no. 1, p. 142, 2008, doi: 10.1186/1471-2180-8-142.
- [58] J. A. Fuentes, N. Villagra, M. Castillo-Ruiz, and G. C. Mora, "The Salmonella Typhi hlyE gene plays a role in invasion of cultured epithelial cells and its functional transfer to S. Typhimurium promotes deep organ infection in mice.," *Res Microbiol*, vol. 159, no. 4, pp. 279–87, May 2008, doi: 10.1016/j.resmic.2008.02.006.
- [59] R. DeSalle, T. T. Sun, T. Bergmann, and A. Garcia-España, "The evolution of tetraspanins through a phylogenetic lens," *Tetraspanins*, pp. 31–45, Jan. 2013, doi: 10.1007/978-94-007-6070-7_2/COVER.
- [60] K. Kitadokoro, "CD81 extracellular domain 3D structure: insight into the tetraspanin superfamily structural motifs," *EMBO J*, vol. 20, no. 1, pp. 12–18, Jan. 2001, doi: 10.1093/emboj/20.1.12.
- [61] B. Zimmerman *et al.*, "Crystal Structure of a Full-Length Human Tetraspanin Reveals a Cholesterol-Binding Pocket," *Cell*, vol. 167, no. 4, pp. 1041–1051.e11, Nov. 2016, doi: 10.1016/J.CELL.2016.09.056.
- [62] T. H. Schmidt, Y. Homsí, and T. Lang, "Oligomerization of the Tetraspanin CD81 via the Flexibility of Its δ -Loop.," *Biophys J*, vol. 110, no. 11, pp. 2463–2474, 2016, doi: 10.1016/j.bpj.2016.05.003.
- [63] Y. Homsí *et al.*, "The Extracellular δ -Domain is Essential for the Formation of CD81 Tetraspanin Webs," *Biophys J*, vol. 107, no. 1, pp. 100–113, Jul. 2014, doi: 10.1016/j.bpj.2014.05.028.
- [64] Y. Homsí and T. Lang, "The specificity of homomeric clustering of CD81 is mediated by its δ -loop," *FEBS Open Bio*, vol. 7, no. 2, pp. 274–283, Feb. 2017, doi: 10.1002/2211-5463.12187.

- [65] M. Palor *et al.*, "Cholesterol sensing by CD81 is important for hepatitis C virus entry," *Journal of Biological Chemistry*, vol. 295, no. 50, pp. 16931–16948, Dec. 2020, doi: 10.1074/jbc.RA120.014761.
- [66] F. Berditchevski *et al.*, "The complexity of tetraspanins," *Biochem Soc Trans*, vol. 39, no. 2, pp. 501–505, Apr. 2011, doi: 10.1042/BST0390501.
- [67] F. Berditchevski and E. Odintsova, "Tetraspanins as regulators of protein trafficking.," *Traffic*, vol. 8, no. 2, pp. 89–96, Feb. 2007, doi: 10.1111/j.1600-0854.2006.00515.x.
- [68] H. T. Maecker, S. C. Todd, and S. Levy, "The tetraspanin superfamily: molecular facilitators.," *FASEB J*, vol. 11, no. 6, pp. 428–42, May 1997.
- [69] E. Rubinstein, "The complexity of tetraspanins," *Biochem Soc Trans*, vol. 39, no. 2, pp. 501–505, Apr. 2011, doi: 10.1042/BST0390501.
- [70] M. Seigneuret, H. Conjeaud, H.-T. Zhang, and X.-P. Kong, "Structural Bases for Tetraspanin Functions," in *Tetraspanins*, Dordrecht: Springer Netherlands, 2013, pp. 1–29. doi: 10.1007/978-94-007-6070-7_1.
- [71] C. Boucheix and E. Rubinstein, "Tetraspanins," *Cellular and Molecular Life Sciences*, vol. 58, no. 9, pp. 1189–1205, Aug. 2001, doi: 10.1007/PL00000933.
- [72] B. Atkinson *et al.*, "Identification of melanoma-associated antigens using fixed tissue screening of antibodies.," *Cancer Res*, vol. 44, no. 6, pp. 2577–81, Jun. 1984.
- [73] R. Oren, S. Takahashi, C. Doss, R. Levy, and S. Levy, "TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins.," *Mol Cell Biol*, vol. 10, no. 8, pp. 4007–15, Aug. 1990, doi: 10.1128/mcb.10.8.4007-4015.1990.
- [74] D. O. Azorsa, J. A. Hyman, and J. E. Hildreth, "CD63/Pltgp40: a platelet activation antigen identical to the stage-specific, melanoma-associated antigen ME491.," *Blood*, vol. 78, no. 2, pp. 280–4, Jul. 1991.
- [75] R. Umeda *et al.*, "Structural insights into tetraspanin CD9 function," *Nat Commun*, vol. 11, no. 1, Dec. 2020, doi: 10.1038/S41467-020-15459-7.
- [76] R. Umeda, T. Nishizawa, and O. Nureki, "Crystallization of the human tetraspanin protein CD9.," *Acta Crystallogr F Struct Biol Commun*, vol. 75, no. Pt 4, pp. 254–259, Apr. 2019, doi: 10.1107/S2053230X1801840X.
- [77] K. J. Susa, S. Rawson, A. C. Kruse, and S. C. Blacklow, "Cryo-EM structure of the B cell co-receptor CD19 bound to the tetraspanin CD81," *Science (1979)*, vol. 371, no. 6526, pp. 300–305, Jan. 2021, doi: 10.1126/science.abd9836.
- [78] C. H. Lipper, K.-H. Gabriel, T. C. M. Seegar, K. L. Dürr, M. G. Tomlinson, and S. C. Blacklow, "Crystal structure of the Tspan15 LEL domain reveals a conserved ADAM10 binding site.," *Structure*, vol. 30, no. 2, pp. 206–214.e4, 2022, doi: 10.1016/j.str.2021.10.007.
- [79] C. S. Stipp, T. v. Kolesnikova, and M. E. Hemler, "Functional domains in tetraspanin proteins," *Trends Biochem Sci*, vol. 28, no. 2, pp. 106–112, Feb. 2003, doi: 10.1016/S0968-0004(02)00014-2.

- [80] M. Zuidscherwoude, F. Göttfert, V. M. E. Dunlock, C. G. Figdor, G. van den Bogaart, and A. B. van Spriel, "The tetraspanin web revisited by super-resolution microscopy," *Sci Rep*, vol. 5, Jul. 2015, doi: 10.1038/SREP12201.
- [81] S. Levy and T. Shoham, "Protein-Protein Interactions in the Tetraspanin Web," *Physiology*, vol. 20, no. 4, pp. 218–224, Aug. 2005, doi: 10.1152/physiol.00015.2005.
- [82] E. Rubinstein, S. Charrin, and M. G. Tomlinson, "Organisation of the Tetraspanin Web," in *Tetraspanins*, Dordrecht: Springer Netherlands, 2013, pp. 47–90. doi: 10.1007/978-94-007-6070-7_3.
- [83] E. Tejera, M. Sala-Valdés, V. Rocha-Perugini, and M. Yáñez-Mó, "The Role of Tetraspanins in Cell Migration and Intercellular Adhesion," *undefined*, pp. 131–167, Jan. 2013, doi: 10.1007/978-94-007-6070-7_6.
- [84] L. Yeung, M. J. Hickey, and M. D. Wright, "The many and varied roles of tetraspanins in immune cell recruitment and migration," *Front Immunol*, vol. 9, no. JUL, p. 1644, Jul. 2018, doi: 10.3389/FIMMU.2018.01644/BIBTEX.
- [85] M. Yáñez-Mó and P. Rousselle, "Generation and characterization of a tetraspanin-decorated reference material for EV research View project Epidermal regeneration View project," 2001. [Online]. Available: <https://www.researchgate.net/publication/12159661>
- [86] R. L. Yauch and M. E. Hemler, "Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase," *Biochemical Journal*, vol. 351, no. Pt 3, p. 629, Nov. 2000, doi: 10.1042/0264-6021:3510629.
- [87] T. Shoham, R. Rajapaksa, C.-C. Kuo, J. Haimovich, and S. Levy, "Building of the Tetraspanin Web: Distinct Structural Domains of CD81 Function in Different Cellular Compartments," *Mol Cell Biol*, vol. 26, no. 4, pp. 1373–1385, Feb. 2006, doi: 10.1128/MCB.26.4.1373-1385.2006.
- [88] V. Carloni, A. Mazzocca, and K. S. Ravichandran, "Tetraspanin CD81 is linked to ERK/MAPKinase signaling by Shc in liver tumor cells," *Oncogene*, vol. 23, no. 8, pp. 1566–1574, Feb. 2004, doi: 10.1038/sj.onc.1207287.
- [89] M. D. Wright, G. W. Moseley, and A. B. van Spriel, "Tetraspanin microdomains in immune cell signalling and malignant disease," *Tissue Antigens*, vol. 64, no. 5, pp. 533–42, Nov. 2004, doi: 10.1111/j.1399-0039.2004.00321.x.
- [90] M. E. Hemler, "Tetraspanin functions and associated microdomains," *Nat Rev Mol Cell Biol*, vol. 6, no. 10, pp. 801–811, Oct. 2005, doi: 10.1038/nrm1736.
- [91] S. Charrin, F. le Naour, O. Silvie, P.-E. Milhiet, C. Boucheix, and E. Rubinstein, "Lateral organization of membrane proteins: tetraspanins spin their web," *Biochemical Journal*, vol. 420, no. 2, pp. 133–154, Jun. 2009, doi: 10.1042/BJ20082422.
- [92] A. B. van Spriel and C. G. Figdor, "The role of tetraspanins in the pathogenesis of infectious diseases," *Microbes Infect*, vol. 12, no. 2, pp. 106–112, Feb. 2010, doi: 10.1016/J.MICINF.2009.11.001.

- [93] T. N. Tham, E. Gouin, E. Rubinstein, C. Boucheix, P. Cossart, and J. Pizarro-Cerda, "Tetraspanin CD81 is required for *Listeria monocytogenes* invasion," *Infect Immun*, vol. 78, no. 1, pp. 204–209, 2010, doi: 10.1128/IAI.00661-09.
- [94] G. References, L. R. Monk, P. N. Partridge, L. J. Morris, P. Gorringe, and A. R. Read, "Investigating the role of tetraspanin proteins in *Salmonella* infection," 2011, doi: 10.13140/RG.2.2.33605.81127.
- [95] L. R. Green, P. N. Monk, L. J. Partridge, P. Morris, A. R. Gorringe, and R. C. Read, "Cooperative role for tetraspanins in adhesin-mediated attachment of bacterial species to human epithelial cells.," *Infect Immun*, vol. 79, no. 6, pp. 2241–9, Jun. 2011, doi: 10.1128/IAI.01354-10.
- [96] L. Cocquerel and O. Silvie, "The Role of CD81 in HCV and *Plasmodium* Infection," in *Tetraspanins*, Dordrecht: Springer Netherlands, 2013, pp. 345–386. doi: 10.1007/978-94-007-6070-7_14.
- [97] O. Silvie *et al.*, "Cholesterol contributes to the organization of tetraspanin-enriched microdomains and to CD81-dependent infection by malaria sporozoites," *J Cell Sci*, vol. 119, no. 10, pp. 1992–2002, May 2006, doi: 10.1242/jcs.02911.
- [98] J. Bruening *et al.*, "Hepatitis C virus enters liver cells using the CD81 receptor complex proteins calpain-5 and CBLB," *PLoS Pathog*, vol. 14, no. 7, p. e1007111, Jul. 2018, doi: 10.1371/journal.ppat.1007111.
- [99] P. Banse *et al.*, "CD81 Receptor Regions outside the Large Extracellular Loop Determine Hepatitis C Virus Entry into Hepatoma Cells," *Viruses*, vol. 10, no. 4, p. 207, Apr. 2018, doi: 10.3390/v10040207.
- [100] C. Bertaux and T. Dragic, "Different Domains of CD81 Mediate Distinct Stages of Hepatitis C Virus Pseudoparticle Entry," *J Virol*, vol. 80, no. 10, pp. 4940–4948, May 2006, doi: 10.1128/JVI.80.10.4940-4948.2006.
- [101] M. Flint *et al.*, "Diverse CD81 Proteins Support Hepatitis C Virus Infection," *J Virol*, vol. 80, no. 22, pp. 11331–11342, Nov. 2006, doi: 10.1128/JVI.00104-06.
- [102] M. Flint *et al.*, "Characterization of Hepatitis C Virus E2 Glycoprotein Interaction with a Putative Cellular Receptor, CD81," *J Virol*, vol. 73, no. 8, pp. 6235–6244, Aug. 1999, doi: 10.1128/JVI.73.8.6235-6244.1999.
- [103] J. Karam, S. Méresse, L. Kremer, and W. Daher, "The roles of tetraspanins in bacterial infections.," *Cell Microbiol*, vol. 22, no. 12, p. e13260, 2020, doi: 10.1111/cmi.13260.
- [104] G. Min, H. Wang, T.-T. Sun, and X.-P. Kong, "Structural basis for tetraspanin functions as revealed by the cryo-EM structure of uroplakin complexes at 6-Å resolution," *Journal of Cell Biology*, vol. 173, no. 6, pp. 975–983, Jun. 2006, doi: 10.1083/jcb.200602086.
- [105] G. Zhou *et al.*, "Uroplakin Ia is the urothelial receptor for uropathogenic *Escherichia coli*: evidence from in vitro FimH binding.," *J Cell Sci*, vol. 114, no. Pt 22, pp. 4095–103, Nov. 2001, doi: 10.1242/jcs.114.22.4095.

- [106] S. Rajesh *et al.*, "Structural basis of ligand interactions of the large extracellular domain of tetraspanin CD81," *J Virol*, vol. 86, no. 18, pp. 9606–16, Sep. 2012, doi: 10.1128/JVI.00559-12.
- [107] N. A. Hassuna, P. N. Monk, F. Ali, R. C. Read, and L. J. Partridge, "A role for the tetraspanin proteins in Salmonella infection of human macrophages," *Journal of Infection*, vol. 75, no. 2, pp. 115–124, Aug. 2017, doi: 10.1016/j.jinf.2017.06.003.
- [108] N. Hassuna, P. N. Monk, G. W. Moseley, and L. J. Partridge, "Strategies for Targeting Tetraspanin Proteins," *BioDrugs*, vol. 23, no. 6, pp. 341–359, Dec. 2009, doi: 10.2165/11315650-000000000-00000.
- [109] J. K. Ventress, L. J. Partridge, R. C. Read, D. Cozens, S. MacNeil, and P. N. Monk, "Peptides from tetraspanin CD9 are potent inhibitors of Staphylococcus aureus adherence to keratinocytes," *PLoS One*, vol. 11, no. 7, Jan. 2016, doi: 10.1371/journal.pone.0160387.
- [110] A. Elgawidi *et al.*, "A role for tetraspanin proteins in regulating fusion induced by Burkholderia thailandensis," *Med Microbiol Immunol*, vol. 209, no. 4, pp. 473–487, Aug. 2020, doi: 10.1007/s00430-020-00670-6.
- [111] T. Sangsri, N. Saiprom, A. Tubsuwan, P. Monk, L. J. Partridge, and N. Chantratita, "Tetraspanins are involved in Burkholderia pseudomallei-induced cell-to-cell fusion of phagocytic and non-phagocytic cells," *Sci Rep*, vol. 10, no. 1, p. 17972, Dec. 2020, doi: 10.1038/S41598-020-74737-Y.
- [112] M. E. Hemler, "Targeting of tetraspanin proteins — potential benefits and strategies," *Nat Rev Drug Discov*, vol. 7, no. 9, pp. 747–758, Sep. 2008, doi: 10.1038/nrd2659.
- [113] R. P. Ramachandran *et al.*, "EspH suppresses erk by spatial segregation from CD81 tetraspanin microdomains," *Infect Immun*, vol. 86, no. 10, Oct. 2018, doi: 10.1128/IAI.00303-18.
- [114] S. M. Roche *et al.*, "Salmonella Typhimurium invalidated for the three currently known invasion factors keeps its ability to invade several cell models," *Front Cell Infect Microbiol*, vol. 8, no. AUG, Aug. 2018, doi: 10.3389/fcimb.2018.00273.
- [115] C. Harman *et al.*, "A View of the E2-CD81 Interface at the Binding Site of a Neutralizing Antibody against Hepatitis C Virus," *J Virol*, vol. 89, no. 1, pp. 492–501, Jan. 2015, doi: 10.1128/JVI.01661-14.
- [116] M. Jamshad *et al.*, "Structural characterization of recombinant human CD81 produced in Pichia pastoris," *Protein Expr Purif*, vol. 57, no. 2, pp. 206–216, Feb. 2008, doi: 10.1016/j.pep.2007.10.013.
- [117] E. Cohen, I. Ophir, and Y. ben Shaul, "Induced differentiation in HT29, a human colon adenocarcinoma cell line," *J Cell Sci*, vol. 112 (Pt 16), no. 16, pp. 2657–2666, 1999, doi: 10.1242/JCS.112.16.2657.
- [118] M. Gagnon, A. Zihler Berner, N. Chervet, C. Chassard, and C. Lacroix, "Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate

- Salmonella adhesion and invasion," *J Microbiol Methods*, vol. 94, no. 3, pp. 274–279, Sep. 2013, doi: 10.1016/j.mimet.2013.06.027.
- [119] M. Palor, L. Stejskal, P. Mandal, A. Shepherd, and J. Grove, "Conformational switching of CD81 controls its function as a receptor for hepatitis C virus," *undefined*, 2019, doi: 10.1101/542837.
- [120] S. Seveau, H. Bierne, S. Giroux, M.-C. Prévost, and P. Cossart, "Role of lipid rafts in E-cadherin- and HGF-R/Met-mediated entry of *Listeria monocytogenes* into host cells," *Journal of Cell Biology*, vol. 166, no. 5, pp. 743–753, Aug. 2004, doi: 10.1083/jcb.200406078.
- [121] F. C. Huang, "Plasma membrane cholesterol plays a critical role in the Salmonella-induced anti-inflammatory response in intestinal epithelial cells," *Cell Immunol*, vol. 271, no. 2, pp. 480–487, 2011, doi: 10.1016/j.cellimm.2011.08.018.
- [122] A. J. M. Santos, M. Meinecke, M. B. Fessler, D. W. Holden, and E. Boucrot, "Preferential invasion of mitotic cells by salmonella reveals that cell surface cholesterol is maximal during metaphase," *J Cell Sci*, vol. 126, no. 14, pp. 2990–2996, Jul. 2013, doi: 10.1242/jcs.115253.
- [123] S. Mahammad and I. Parmryd, "Cholesterol Depletion Using Methyl- β -cyclodextrin," 2015, pp. 91–102. doi: 10.1007/978-1-4939-1752-5_8.
- [124] A. H. Hinzey *et al.*, "Choice of cyclodextrin for cellular cholesterol depletion for vascular endothelial cell lipid raft studies: cell membrane alterations, cytoskeletal reorganization and cytotoxicity," *Indian J Biochem Biophys*, vol. 49, no. 5, pp. 329–41, Oct. 2012.
- [125] A. Higginbottom *et al.*, "Identification of Amino Acid Residues in CD81 Critical for Interaction with Hepatitis C Virus Envelope Glycoprotein E2," *J Virol*, vol. 74, no. 8, pp. 3642–3649, Apr. 2000, doi: 10.1128/JVI.74.8.3642-3649.2000.
- [126] T. Krey *et al.*, "The disulfide bonds in glycoprotein E2 of hepatitis C virus reveal the tertiary organization of the molecule," *PLoS Pathog*, vol. 6, no. 2, Feb. 2010, doi: 10.1371/journal.ppat.1000762.
- [127] R. S. Dize, M. R. Frey, R. H. Whitehead, and D. B. Polk, "Epidermal growth factor stimulates Rac activation through Src and phosphatidylinositol 3-kinase to promote colonic epithelial cell migration," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 294, no. 1, pp. G276–G285, Jan. 2008, doi: 10.1152/ajpgi.00340.2007.
- [128] R. E. Itoh, E. Kiyokawa, K. Aoki, T. Nishioka, T. Akiyama, and M. Matsuda, "Phosphorylation and activation of the Rac1 and Cdc42 GEF Asef in A431 cells stimulated by EGF," *J Cell Sci*, vol. 121, no. 16, pp. 2635–2642, Aug. 2008, doi: 10.1242/jcs.028647.
- [129] C. Chiasson-MacKenzie and A. I. McClatchey, "EGFR-induced cytoskeletal changes drive complex cell behaviors: The tip of the iceberg," *Sci Signal*, vol. 11, no. 515, Jan. 2018, doi: 10.1126/scisignal.aas9473.

- [130] A. Bolshakova, K. E. Magnusson, G. Pinaev, and O. Petukhova, "EGF-induced dynamics of NF- κ B and F-actin in A431 cells spread on fibronectin," *Histochem Cell Biol*, vol. 144, no. 3, pp. 223–235, Sep. 2015, doi: 10.1007/s00418-015-1331-5.
- [131] P. Dean, L. Young, S. Quitard, and B. Kenny, "Insights into the Pathogenesis of Enteropathogenic *E. coli* Using an Improved Intestinal Enterocyte Model," *PLoS One*, vol. 8, no. 1, p. e55284, Jan. 2013, doi: 10.1371/journal.pone.0055284.
- [132] E. Wagner *et al.*, "Virulence characterization and comparative genomics of *Listeria monocytogenes* sequence type 155 strains," *BMC Genomics*, vol. 21, no. 1, Dec. 2020, doi: 10.1186/S12864-020-07263-W.
- [133] N. E. Zeitouni, P. Dersch, H. Y. Naim, and M. von K  ckritz-Blickwede, "Hypoxia Decreases Invasin-Mediated *Yersinia enterocolitica* Internalization into Caco-2 Cells," *PLoS One*, vol. 11, no. 1, p. e0146103, Jan. 2016, doi: 10.1371/journal.pone.0146103.
- [134] T. Lea, "Caco-2 Cell Line," in *The Impact of Food Bioactives on Health*, Cham: Springer International Publishing, 2015, pp. 103–111. doi: 10.1007/978-3-319-16104-4_10.
- [135] Y. Sambuy, I. de Angelis, G. Ranaldi, M. L. Scarino, A. Stammati, and F. Zucco, "The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics," *Cell Biol Toxicol*, vol. 21, no. 1, pp. 1–26, Jan. 2005, doi: 10.1007/s10565-005-0085-6.
- [136] F. Delie and W. Rubas, "A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model," *Crit Rev Ther Drug Carrier Syst*, vol. 14, no. 3, pp. 221–86, 1997.
- [137] I. Lesuffleur, A. Barbai, E. Dussaulx, and A. Zweibaum, "Growth Adaptation to Methotrexate of HT-29 Human Colon Carcinoma Cells Is Associated with Their Ability to Differentiate into Columnar Absorptive and Mucus-secreting Cells'," 1990.
- [138] M. T. Cairns, A. Gupta, J. A. Naughton, M. Kane, M. Clyne, and L. Joshi, "Glycosylation-related gene expression in HT29-MTX-E12 cells upon infection by *Helicobacter pylori*," *World J Gastroenterol*, vol. 23, no. 37, pp. 6817–6832, Oct. 2017, doi: 10.3748/wjg.v23.i37.6817.
- [139] X. Li, N. M. C. Bleumink-Pluym, Y. M. C. A. Luijkx, R. W. Wubbolts, J. P. M. van Putten, and K. Strijbis, "MUC1 is a receptor for the *Salmonella* SiiE adhesin that enables apical invasion into enterocytes," *PLoS Pathog*, vol. 15, no. 2, p. e1007566, Feb. 2019, doi: 10.1371/journal.ppat.1007566.
- [140] P. Hoffmann *et al.*, "Caco-2/HT29-MTX co-cultured cells as a model for studying physiological properties and toxin-induced effects on intestinal cells," *PLoS One*, vol. 16, no. 10, p. e0257824, Oct. 2021, doi: 10.1371/journal.pone.0257824.
- [141] M. Gagnon, A. Zihler Berner, N. Chervet, C. Chassard, and C. Lacroix, "Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate *Salmonella* adhesion and invasion," *J Microbiol Methods*, vol. 94, no. 3, pp. 274–279, Sep. 2013, doi: 10.1016/j.mimet.2013.06.027.

- [142] J. M. Laparra and Y. Sanz, "Comparison of *in vitro* models to study bacterial adhesion to the intestinal epithelium," *Lett Appl Microbiol*, vol. 49, no. 6, pp. 695–701, Dec. 2009, doi: 10.1111/j.1472-765X.2009.02729.x.
- [143] S. Charrin *et al.*, "The major CD9 and CD81 molecular partner. Identification and characterization of the complexes," *Journal of Biological Chemistry*, vol. 276, no. 17, pp. 14329–14337, 2001, doi: 10.1074/jbc.M011297200.
- [144] F. le Naour *et al.*, "Profiling of the Tetraspanin Web of Human Colon Cancer Cells," *Molecular & Cellular Proteomics*, vol. 5, no. 5, pp. 845–857, May 2006, doi: 10.1074/mcp.M500330-MCP200.
- [145] S. Bagchi, R. Fredriksson, and Å. Wallén-Mackenzie, "In Situ Proximity Ligation Assay (PLA)," 2015, pp. 149–159. doi: 10.1007/978-1-4939-2742-5_15.
- [146] L. A. Knodler, V. Nair, and O. Steele-Mortimer, "Quantitative assessment of cytosolic Salmonella in epithelial cells," *PLoS One*, vol. 9, no. 1, Jan. 2014, doi: 10.1371/JOURNAL.PONE.0084681.
- [147] A. B. van Spriel and C. G. Figdor, "The role of tetraspanins in the pathogenesis of infectious diseases," *Microbes Infect*, vol. 12, no. 2, pp. 106–112, Feb. 2010, doi: 10.1016/J.MICINF.2009.11.001.
- [148] L. A. Fast *et al.*, "Inhibition of tetraspanin functions impairs human papillomavirus and cytomegalovirus infections," *Int J Mol Sci*, vol. 19, no. 10, Oct. 2018, doi: 10.3390/ijms19103007.
- [149] L. Lasswitz *et al.*, "The Tetraspanin CD81 Is a Host Factor for Chikungunya Virus Replication," *mBio*, vol. 13, no. 3, Jun. 2022, doi: 10.1128/mbio.00731-22.
- [150] J. K. Ventress, L. J. Partridge, R. C. Read, D. Cozens, S. MacNeil, and P. N. Monk, "Peptides from tetraspanin CD9 are potent inhibitors of Staphylococcus aureus adherence to keratinocytes," *PLoS One*, vol. 11, no. 7, Jan. 2016, doi: 10.1371/JOURNAL.PONE.0160387.
- [151] O. Silvie *et al.*, "Cholesterol contributes to the organization of tetraspanin-enriched microdomains and to CD81-dependent infection by malaria sporozoites," *J Cell Sci*, vol. 119, no. 10, pp. 1992–2002, May 2006, doi: 10.1242/jcs.02911.
- [152] S. Charrin, S. Jouannet, C. Boucheix, and E. Rubinstein, "Tetraspanins at a glance," *J Cell Sci*, Jan. 2014, doi: 10.1242/jcs.154906.
- [153] P. N. Monk and L. J. Partridge, "Tetraspanins-Gateways for Infection," 2012.
- [154] I. Vasiliauskaite *et al.*, "Conformational Flexibility in the Immunoglobulin-Like Domain of the Hepatitis C Virus Glycoprotein E2," *mBio*, vol. 8, no. 3, May 2017, doi: 10.1128/MBIO.00382-17.
- [155] G. Koutsoudakis, E. Herrmann, S. Kallis, R. Bartenschlager, and T. Pietschmann, "The Level of CD81 Cell Surface Expression Is a Key Determinant for Productive Entry of Hepatitis C Virus into Host Cells," *J Virol*, vol. 81, no. 2, pp. 588–598, Jan. 2007, doi: 10.1128/JVI.01534-06.

- [156] X. Deng *et al.*, "Laboratory-based surveillance of non-typhoidal *Salmonella* infections in Guangdong Province, China," *Foodborne Pathog Dis*, vol. 9, no. 4, pp. 305–312, Apr. 2012, doi: 10.1089/FPD.2011.1008.
- [157] L. Mijouin *et al.*, "*Salmonella enteritidis* Rck-mediated invasion requires activation of Rac1, which is dependent on the class I PI 3-kinases-Akt signaling pathway," *The FASEB Journal*, vol. 26, no. 4, pp. 1569–1581, Apr. 2012, doi: 10.1096/fj.11-189647.
- [158] B. Michael, J. N. Smith, S. Swift, F. Heffron, and B. M. M. Ahmer, "SdiA of *Salmonella enterica* Is a LuxR Homolog That Detects Mixed Microbial Communities," *J Bacteriol*, vol. 183, no. 19, pp. 5733–5742, Oct. 2001, doi: 10.1128/JB.183.19.5733-5742.2001.
- [159] F. Habyarimana, A. Sabag-Daigle, and B. M. M. Ahmer, "The SdiA-Regulated Gene *srgE* Encodes a Type III Secreted Effector," *J Bacteriol*, vol. 196, no. 12, pp. 2301–2312, Jun. 2014, doi: 10.1128/JB.01602-14.
- [160] S. A. Fattinger *et al.*, "*Salmonella* Typhimurium discreet-invasion of the murine gut absorptive epithelium," *PLoS Pathog*, vol. 16, no. 5, May 2020, doi: 10.1371/journal.ppat.1008503.
- [161] M. Yáñez-Mó, O. Barreiro, M. Gordon-Alonso, M. Sala-Valdés, and F. Sánchez-Madrid, "Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes," *Trends in Cell Biology*, vol. 19, no. 9, pp. 434–446, Sep. 2009, doi: 10.1016/j.tcb.2009.06.004.
- [162] G. P. Coffey *et al.*, "Engagement of CD81 induces ezrin tyrosine phosphorylation and its cellular redistribution with filamentous actin," *J Cell Sci*, vol. 122, no. 17, pp. 3137–3144, Sep. 2009, doi: 10.1242/jcs.045658.
- [163] R. Bari *et al.*, "Tetraspanins regulate the protrusive activities of cell membrane," *Biochem Biophys Res Commun*, vol. 415, no. 4, pp. 619–626, Dec. 2011, doi: 10.1016/j.bbrc.2011.10.121.
- [164] T. Quast *et al.*, "CD81 is essential for the formation of membrane protrusions and regulates Rac1-activation in adhesion-dependent immune cell migration.," *Blood*, vol. 118, no. 7, pp. 1818–1827, Jun. 2011, doi: 10.1182/BLOOD-2010-12-326595.
- [165] E. Tejera *et al.*, "CD81 regulates cell migration through its association with Rac GTPase," *Mol Biol Cell*, vol. 24, no. 3, pp. 261–273, Jan. 2013, doi: 10.1091/MBC.E12-09-0642.
- [166] C. Phoebe Lostroh and C. A. Lee, "The *Salmonella* pathogenicity island-1 type III secretion system," *Microbes Infect*, vol. 3, no. 14–15, pp. 1281–1291, Nov. 2001, doi: 10.1016/S1286-4579(01)01488-5.
- [167] Y. Homsy *et al.*, "The extracellular δ -domain is essential for the formation of CD81 tetraspanin webs," *Biophys J*, vol. 107, no. 1, pp. 100–113, Jul. 2014, doi: 10.1016/j.bpj.2014.05.028.
- [168] G. Vieyres, X. Thomas, V. Descamps, G. Duverlie, A. H. Patel, and J. Dubuisson, "Characterization of the envelope glycoproteins associated with infectious hepatitis C virus," *J Virol*, vol. 84, no. 19, pp. 10159–10168, Oct. 2010, doi: 10.1128/JVI.01180-10.

- [169] I. Vasiliauskaite, "Structural characterization of viral envelope glycoproteins," 2014. Accessed: Nov. 08, 2022. [Online]. Available: <https://tel.archives-ouvertes.fr/tel-01230548>
- [170] F. C. Huang, "The critical role of membrane cholesterol in Salmonella-induced autophagy in intestinal epithelial cells," *Int J Mol Sci*, vol. 15, no. 7, pp. 12558–12572, Jul. 2014, doi: 10.3390/ijms150712558.
- [171] F. C. Huang, "Plasma membrane cholesterol plays a critical role in the Salmonella-induced anti-inflammatory response in intestinal epithelial cells," *Cell Immunol*, vol. 271, no. 2, pp. 480–487, 2011, doi: 10.1016/j.cellimm.2011.08.018.
- [172] S. Charrin *et al.*, "A physical and functional link between cholesterol and tetraspanins," *Eur J Immunol*, vol. 33, no. 9, pp. 2479–2489, Sep. 2003, doi: 10.1002/eji.200323884.
- [173] M. Palor *et al.*, "Cholesterol sensing by CD81 is important for hepatitis C virus entry," *Journal of Biological Chemistry*, vol. 295, no. 50, pp. 16931–16948, Dec. 2020, doi: 10.1074/JBC.RA120.014761/ATTACHMENT/058083F8-CA16-4CCA-AE1C-020BA4677C09/MMC1.ZIP.
- [174] S. Charrin *et al.*, "The Major CD9 and CD81 Molecular Partner," *Journal of Biological Chemistry*, vol. 276, no. 17, pp. 14329–14337, Apr. 2001, doi: 10.1074/jbc.M011297200.
- [175] E. Gustafson-Wagner and C. S. Stipp, "The CD9/CD81 Tetraspanin Complex and Tetraspanin CD151 Regulate $\alpha 3\beta 1$ Integrin-Dependent Tumor Cell Behaviors by Overlapping but Distinct Mechanisms," *PLoS One*, vol. 8, no. 4, p. e61834, Apr. 2013, doi: 10.1371/journal.pone.0061834.
- [176] M. Frolikova *et al.*, "CD9 and CD81 Interactions and Their Structural Modelling in Sperm Prior to Fertilization," *Int J Mol Sci*, vol. 19, no. 4, p. 1236, Apr. 2018, doi: 10.3390/ijms19041236.
- [177] J. Franz *et al.*, "Nanoscale Imaging Reveals a Tetraspanin-CD9 Coordinated Elevation of Endothelial ICAM-1 Clusters," *PLoS One*, vol. 11, no. 1, p. e0146598, Jan. 2016, doi: 10.1371/journal.pone.0146598.
- [178] R. Reyes, B. Cardeñes, Y. Machado-Pineda, and C. Cabañas, "Tetraspanin CD9: A Key Regulator of Cell Adhesion in the Immune System," *Front Immunol*, vol. 9, no. APR, Apr. 2018, doi: 10.3389/FIMMU.2018.00863.
- [179] M. Lelek *et al.*, "Single-molecule localization microscopy," *Nature Reviews Methods Primers*, vol. 1, no. 1, p. 39, Jun. 2021, doi: 10.1038/s43586-021-00038-x.
- [180] Y. Takeda *et al.*, "Tetraspanins CD9 and CD81 function to prevent the fusion of mononuclear phagocytes," *J Cell Biol*, vol. 161, no. 5, pp. 945–956, Jun. 2003, doi: 10.1083/JCB.200212031.
- [181] K. Miyado *et al.*, "Requirement of CD9 on the Egg Plasma Membrane for Fertilization," *Science (1979)*, vol. 287, no. 5451, pp. 321–324, Jan. 2000, doi: 10.1126/science.287.5451.321.
- [182] M. Fanaei, P. N. Monk, and L. J. Partridge, "The role of tetraspanins in fusion," *Biochem Soc Trans*, vol. 39, no. 2, pp. 524–528, 2011, doi: 10.1042/BST0390524.

- [183] J. P. Gorvel and S. Méresse, "Maturation steps of the Salmonella-containing vacuole," *Microbes Infect*, vol. 3, no. 14–15, pp. 1299–1303, 2001, doi: 10.1016/S1286-4579(01)01490-3.
- [184] J. Fredlund *et al.*, "The entry of Salmonella in a distinct tight compartment revealed at high temporal and ultrastructural resolution," *Cell Microbiol*, vol. 20, no. 4, Apr. 2018, doi: 10.1111/cmi.12816.
- [185] V. Stévenin *et al.*, "Dynamic Growth and Shrinkage of the Salmonella-Containing Vacuole Determines the Intracellular Pathogen Niche," *Cell Rep*, vol. 29, no. 12, pp. 3958–3973.e7, Dec. 2019, doi: 10.1016/j.celrep.2019.11.049.
- [186] M. E. Hemler, "Tetraspanin Proteins Mediate Cellular Penetration, Invasion, and Fusion Events and Define a Novel Type of Membrane Microdomain," in *Annual Review of Cell and Developmental Biology*, 2003, pp. 397–422. doi: 10.1146/annurev.cellbio.19.111301.153609.
- [187] X. A. Zhang and C. Huang, "Tetraspanins and cell membrane tubular structures," *Cellular and Molecular Life Sciences*, vol. 69, no. 17, pp. 2843–2852, Mar. 2012, doi: 10.1007/s00018-012-0954-0.
- [188] K. Artavanis-Tsakonas *et al.*, "The Tetraspanin CD82 Is Specifically Recruited to Fungal and Bacterial Phagosomes prior to Acidification," *Infect Immun*, vol. 79, no. 3, pp. 1098–1106, Mar. 2011, doi: 10.1128/IAI.01135-10.
- [189] M. J. Martínez-Lorenzo, S. Méresse, C. de Chastellier, and J. P. Gorvel, "Unusual intracellular trafficking of Salmonella typhimurium in human melanoma cells," *Cell Microbiol*, vol. 3, no. 6, pp. 407–416, 2001, doi: 10.1046/j.1462-5822.2001.00123.x.
- [190] S. Huang *et al.*, "The phylogenetic analysis of tetraspanins projects the evolution of cell–cell interactions from unicellular to multicellular organisms," *Genomics*, vol. 86, no. 6, pp. 674–684, Dec. 2005, doi: 10.1016/j.ygeno.2005.08.004.
- [191] Z. Liu, H. Shi, A. K. Nzessi, A. Norris, B. D. Grant, and J. Liu, "Tetraspanins TSP-12 and TSP-14 function redundantly to regulate the trafficking of the type II BMP receptor in *Caenorhabditis elegans*," *Proceedings of the National Academy of Sciences*, vol. 117, no. 6, pp. 2968–2977, Feb. 2020, doi: 10.1073/pnas.1918807117.
- [192] Y. Gao, C. Spahn, M. Heilemann, and L. J. Kenney, "The pearling transition provides evidence of force-driven endosomal tubulation during Salmonella infection," *mBio*, vol. 9, no. 3, May 2018, doi: 10.1128/mBio.01083-18.
- [193] S. A. Martins, P. D. Correia, R. A. Dias, O. A. B. da Cruz e Silva, and S. I. Vieira, "CD81 Promotes a Migratory Phenotype in Neuronal-Like Cells," *Microscopy and Microanalysis*, vol. 25, no. 1, pp. 229–235, Feb. 2019, doi: 10.1017/S1431927618015532.
- [194] A. K. Criss, D. M. Ahlgren, T. S. Jou, B. A. McCormick, and J. E. Casanova, "The GTPase Rac1 selectively regulates Salmonella invasion at the apical plasma membrane of polarized epithelial cells," *J Cell Sci*, vol. 114, no. Pt 7, pp. 1331–1341, 2001, doi: 10.1242/JCS.114.7.1331.

- [195] K. Ehrbar, S. Miold, A. Friebe, S. Stender, and W.-D. Hardt, "Characterization of effector proteins translocated via the SPI1 type III secretion system of *Salmonella typhimurium*," *International Journal of Medical Microbiology*, vol. 291, no. 6–7, pp. 479–485, Jan. 2001, doi: 10.1078/1438-4221-00156.
- [196] A. Friebe, H. Ilchmann, M. Aepfelbacher, K. Ehrbar, W. Machleidt, and W. D. Hardt, "SopE and SopE2 from *Salmonella typhimurium* Activate Different Sets of RhoGTPases of the Host Cell," *Journal of Biological Chemistry*, vol. 276, no. 36, pp. 34035–34040, Sep. 2001, doi: 10.1074/jbc.M100609200.
- [197] L. Mijouin *et al.*, "Salmonella enteritidis Rck-mediated invasion requires activation of Rac1, which is dependent on the class I PI 3-kinases-Akt signaling pathway," *The FASEB Journal*, vol. 26, no. 4, pp. 1569–1581, Apr. 2012, doi: 10.1096/fj.11-189647.
- [198] F. Vences-Catalán *et al.*, "Targeting the tetraspanin CD81 reduces cancer invasion and metastasis," *Proc Natl Acad Sci U S A*, vol. 118, no. 24, p. e2018961118, Jun. 2021, doi: 10.1073/PNAS.2018961118/SUPPL_FILE/PNAS.2018961118.SAPP.PDF.
- [199] J. Zhang, G. Randall, A. Higginbottom, P. Monk, C. M. Rice, and J. A. McKeating, "CD81 is required for hepatitis C virus glycoprotein-mediated viral infection," *J Virol*, vol. 78, no. 3, pp. 1448–1455, Feb. 2004, doi: 10.1128/JVI.78.3.1448-1455.2004.
- [200] T. Dull *et al.*, "A third-generation lentivirus vector with a conditional packaging system," *J Virol*, vol. 72, no. 11, pp. 8463–71, Nov. 1998, doi: 10.1128/JVI.72.11.8463-8471.1998.
- [201] N. E. Sanjana, O. Shalem, and F. Zhang, "Improved vectors and genome-wide libraries for CRISPR screening," *Nat Methods*, vol. 11, no. 8, pp. 783–784, Aug. 2014, doi: 10.1038/nmeth.3047.
- [202] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001, doi: 10.1006/METH.2001.1262.
- [203] T. Krey *et al.*, "The Disulfide Bonds in Glycoprotein E2 of Hepatitis C Virus Reveal the Tertiary Organization of the Molecule," *PLoS Pathog*, vol. 6, no. 2, p. e1000762, Feb. 2010, doi: 10.1371/journal.ppat.1000762.
- [204] J. Schindelin *et al.*, "Fiji: an open-source platform for biological-image analysis," *Nat Methods*, vol. 9, no. 7, pp. 676–682, Jul. 2012, doi: 10.1038/NMETH.2019.
- [205] M. Seigneuret, "Complete Predicted Three-Dimensional Structure of the Facilitator Transmembrane Protein and Hepatitis C Virus Receptor CD81: Conserved and Variable Structural Domains in the Tetraspanin Superfamily," *Biophys J*, vol. 90, no. 1, pp. 212–227, Jan. 2006, doi: 10.1529/biophysj.105.069666.

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I dedicate this work to all the people who enjoy doing science as much as I do, especially the ones who find joy in understanding the intricacies and the intelligence of the microbial world. 🌱

Maraming salamat po sa inyong lahat!

Declaration

Herewith, I confirm that I have written the present PhD thesis myself and independently in compliance with “the policy of Hannover Medical School on the safeguarding of good scientific practice and procedural rules for dealing with scientific misconduct” and that I have not submitted it at any other university worldwide.

Herewith, I agree that MHH can check my thesis by plagiarism detection software as well as randomly check the primary data. I am aware that in case of suspicion, ombudsman proceedings according to § 9 of MHH ‘Guidelines of Hannover Medical School to guarantee good scientific practice and dealing with scientific fraud’ will be initiated. During such proceedings, the PhD process is paused.

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Profile Summary:

Microbiologist with a deep interest on host-pathogen interactions and pathogen biology. Highly skilled on using and creating molecular tools to investigate host-pathogen interactions. Easy-going, always optimistic and an innovative self-starter, with proven accomplishments in handling cross-functional roles and high enthusiasm for collaborative pursuits across teams.

Key Skills and Methods Expertise

- *Genetic engineering/Synthetic Biology*: CRISPR-based gene editing of mammalian cells · lentiviral transduction · molecular cloning methods to generate tagged fusion proteins · transfection experiments · nucleic acid extraction and purification techniques · PCR methods/primer design · qPCR (Bio-Rad) · Sanger sequencing (ABI 3130) and basic bioinformatic tools (*i.e.*, SnapGene, ApE, Chromas, BLAST/NCBI)
- *Protein-based methods*: recombinant protein production and purification in bacteria · western blot detection.
- *Microscopy and in vitro assays*: flow cytometry · laser confocal microscopy and widefield epifluorescent systems (Zeiss, Leica and Nikon microscopes) · live cell imaging experiments (Nikon system) including image data analysis (ImageJ, Leica LASX, Zen Blue, Metamorph)
- *Infection experiments* on cell culture and host-derived intestinal organoid systems (mouse and human).
- Compendial-based analytical microbiological methods and standard microbiological methods and familiarity with GMP, HACCP and ISO-based standards.
- Data analysis and presentation using software packages (*i.e.* GraphPad Prism, BioRender, Microsoft Office tools)
- Collaboration with different institutes and core facilities to execute research plans and activities.
- Technical communication of scientific data to academic audience and general audience.
- Effective teaching/demonstration strategies for scientific concepts, instrumentations, and laboratory techniques for basic to advanced microbiology training for undergraduate students.
- Strong business/entrepreneurial mindset with experience in building business relationships with an IVD company based on a co-founded start-up.

WORK EXPERIENCE

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2. Implemented adoption of, and development of new assay and experimental methodologies (proximity labeling, CRISPR techniques, lentiviral transduction, nucleofection, laser confocal imaging techniques, infection assay techniques etc) for use within the project and adoption by other members within the laboratory work group.

	- Coordinated with the principal investigator and fostered collaborations across different work groups and core facilities to carry out research activities within targeted timelines and project duration. - Mentored and trained masters' and bachelors' students to properly execute thesis experiments and data analysis. - Delivered oral and poster presentations of current research findings at international conferences and seminars.
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July 2015 to July 2016	Research Fellowship (July 2015 – July 2016) <i>Biomolecular Science and Engineering Laboratory/ Nagai Lab (home page)</i> <i>Institute for Scientific and Industrial Research</i> <i>Osaka University, Japan</i> Worked on developing a <i>Pseudomonas aeruginosa</i> -based vector expressing secretable fusion proteins for potential theranostic applications <i>i.e.</i> tumor-targeting bacterial vector.
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PROFESSIONAL ORGANIZATIONS

- German Society for Microbiology (DGHM)
- International Association for Food Protection (IAFP)

Other past functions

July 2010 – June 2011	Quality Control Microbiologist (<i>sterile parenteral drugs</i>) Euro-med Laboratories, Philippines, Incorporated
May 2011	Certified Registered Microbiologist Philippine Academy of Microbiology
Nov 2009 – May 2010	Sales Associate- Professional Diagnostics (<i>IVD assays/kits</i>) Roche (Philippines) Incorporated
May 2009 – Oct 2009	Quality Control - Laboratory Analyst (<i>sterile medical devices</i>) Terumo (Philippines) Corporation
April - May 2008	Quality Control Laboratory Intern (<i>veterinary pharmaceuticals</i>) Animal Healthcare Bayer (Philippines) Incorporated

Other technical/entrepreneurial ventures

Co-Founder, MicroCore Review Center (2022- present) – online digital learning platform providing coaching and lecture reviews to students preparing for the certification exam for Registered Microbiologists administered by the Philippine Academy for Microbiology

EDUCATIONAL BACKGROUND

Doctor of Philosophy in Infection Biology/Microbiology (2018-2023)

*Hannover Medical School, Germany
Institute of Medical Microbiology and Hospital Epidemiology
German Center for Infection Research (DZIF)*

Thesis: Investigation of the role of host tetraspanins CD81 and CD9 during *Salmonella* invasion

Final grade: *Magna cum laude* (defended January 20, 2023)

Supervisor: Prof. Dr. Guntram A. Grassl (grassl.guntram@mh-hannover.de)

Co-Supervisors: Prof. Dr. Gisa Gerold (Veterinary Medical School, Hannover/ Umea University, Sweden)
Dr. Agnes Wiedemann (University of Toulouse/IRSD, INRAE, France)

Funding: German Academic Exchange Fellowship (DAAD), SFB900 Chronic Infections

Graduate Research Fellowship in Biotechnology (2015- 2016)

*Osaka University, Japan
Institute for Scientific and Industrial Research /International Center for Biotechnology*

Project: Genetic engineering of *Pseudomonas aeruginosa* for molecular theranostics applications

Supervisor: Prof. Takeharu Nagai (nagai@sanken.osaka-u.ac.jp)

Funding: UNESCO Biotechnology School in Asia Fellowship

Master of Science in Microbiology minor in Food Science (2012-2017)

*University of the Philippines, Los Baños
Microbiology Division, Institute of Biological Sciences*

Thesis: Fluorescent protein labelling of *Pseudomonas aeruginosa* Type III secretion effectors for host infection studies and biomedical applications

Supervisor: Emeritus Professor, Ida Dalmacio (ifdalmacio@up.edu.ph)

Awards/Distinction

2017 GapSummit- 100 Leaders of Tomorrow (Georgetown University, Washington D.C., USA)

2015 Thesis subsidy grant award - Philippine Society for Microbiology, Inc.

2015 Thesis support grant- 3M Food Safety (Philippines)

2014 Travel grant award - International Association for Food Protection (Indianapolis, Indiana USA)

Bachelor of Science in Biology major in Microbiology (2005- 2009)

University of the Philippines, Los Baños

Awards/Distinction: Phi Sigma Biological Sciences Honours Society

Hobbies/other interests: Films, performance arts, cooking Asian desserts and cuisine, biking, travelling and exploring other cultures.