The Role of *Salmonella* Pathogenicity Island-2 (SPI-2) in the Course of Neonatal Non-typhoidal *Salmonella* Infections

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the

Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by

Kira van Vorst

2019

This study was conducted from March 2015 to July 2016 at the Institute of Microbiology of the University of Veterinary Medicine Hannover and continued from July 2016 to October 2019 at the Institute of Microbiology and Epizootics, Department of Veterinary Medicine of the Freie Universität Berlin under supervision of Prof. Dr. Marcus Fulde.

1st supervisor: Prof. Dr. Marcus Fulde, PhD

2nd supervisor: Prof. Dr. Haike Antelmann

Date of final exam: 06.02.2020

Meiner Mutter, der ich alles verdanke

Content

Abbreviations

°C	Celsius
µl	microliter, 10^{-6} liters
µm	micrometer, 10^{-6} meter
A	adenine (nucleobase)
a.a.	amino acid uptake + Δ*sseB* mutant
AMP	ampicillin
BMMs	bone marrow macrophages
BSA	bovine serum albumin
c	concentration
CFU	colony forming units
CM	chloramphenicol
ctrl.	control
Cxcl2	C-X-C motif chemokine 2, murine gene
Cxcl5	C-X-C motif chemokine 5, murine gene
DC	dendritic cell
DNA	deoxyribonucleic acid
dpi	days post infection
E. coli	*Escherichia coli*
e.g.	exempli gratia
EDTA	Ethylenediaminetetraacetic acid
EM	electronmicroscopic pictures
f.a.	fatty acid uptake + Δ*sseB* mutant
FCS	fetal calve serum
g	gram
gDNA	genomic DNA
GFP	green fluorescent protein
gluc.	glucose and glucose-6-phosphate uptake + Δ*sseB* mutant
H	flagella antigen
HIV	human immunodeficiency virus
Hprt	Hypoxanthine Phosphoribosyltransferase, murine gene
i.p.	intraperitoneal

i.v.	intravenous
IECs	intestinal epithelial cells
IL	interleukin
ILFs	isolated lymphoid follicles
iNTS	invasive non-typhoidal *Salmonella*
K	capsular antigen
KAN	kanamycin
KO	knockout
L	liter
LAMP-1	Lysosomal-associated membrane protein 1, murine
LPS	lipopolysaccharide
M cells	microfold cells
MAPK	mitogen-activated protein kinase
min	minutes
ml	milliliter
MLN	mesenteric lymph node
mM	millimolar, 10^{-3} mol/L
mRNA	messenger ribonucleic acid
MTOC	microtubule-organizing center
Myd88	myeloid differentiation primary response 88, murine gene
MYD88	myeloid differentiation primary response 88, murine protein
N	nucleus
nd	not determined
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
no.	number
Nos2	inducible nitric oxide synthase 2, murine gene
NRAMP1	murine natural resistance-associated macrophage protein 1
ns	not significant, $p \geq 0.05$
NTS	non-typhoidal *Salmonella*
O	somatic antigen
p.i.	post infection
p.p.	*post partum*
PAMP	pathogen-associated molecular pattern

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pep.	peptide uptake + Δ*sseB* mutant
pfa	paraformaldehyde
PI	pathogenicity island
PMN	polymorphonuclear leukocyte
PRR	pattern recognition receptor
RegIIIγ	regenerating islet-derived protein 3 gamma, , murine gene
RILP	Rab-interacting lysosomal protein
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rotations per minute
rRNA	risbosomal ribonucleic acid
RT	room temperature
RT PCR	real-time PCR
S. bongori	*Salmonella bongori*
S. enterica	*Salmonella enterica*
S. Typhi	*Salmonella enterica* subsp. *enterica* serovar Typhi
Saa3	serum amyloid A 3, murine gene
SCV	*Salmonella*-containing vacuole
sec	seconds
ser.	serovar/serotype
SI	small intestine
SIFs	*Salmonella*-induced filaments
SKIP	SifA and kinesin-interacting protein
Slc11A1	Solute Carrier Family 11 Member 1, murine gene
SPI	*Salmonella*-pathogenicity island
spp.	Species pluralis
STy	*Salmonella enterica* subsp. *enterica* serovar Typhimurium
subsp.	subspecies
T	thymine (nucleobase)
T3SS	type three secretion system
T3SS-2	Type-three secretion system of *Salmonella*-pathogenicity island-2

TEM	transelectron microscopy
TLR	Toll-like receptor, murine
VFE	vacuolated foetal enterocytes
vs	versus
WITS	wild-type isogenic tagged *Salmonella*
wt	wild type
x g	times gravity

List of figures

List of tables

Summary

As a major cause of infectious diarrheal diseases, non-typhoidal *Salmonella* (NTS), such as *S. enterica* subsp. *enterica* serovar Typhimurium, are of global health concern for both human and veterinary medicine. Besides causing usually self-limiting gastroenteritis in immunocompetent adult individuals, infection of neonates with this pathogen can induce invasive and potentially life-threatening conditions. Invasive infection caused by NTS are predominantly observed in developing countries with poor hygiene standards, where the pathogen is frequently isolated from cases of neonatal sepsis and meningitis. The pathogenicity of *Salmonella* is conferred by certain virulence factors encoded on chromosomal regions acquired by horizontal gene transfer, called *Salmonella* pathogenicity islands (SPIs). Delivery of effector proteins of SPI-1 and SPI-2 into the host cell is enabled via own type three secretion systems (T3SSs). The role of SPI effectors in host-pathogen interactions has been extensively studied *in vitro* and it is widely accepted that SPI-2 is crucial for the establishment of an intracellular compartment, the *Salmonella*-containing vacuole (SCV), which allows the bacteria to survive and replicate inside the host cell. Besides this, the translocated effector proteins allow the pathogen to modulate and manipulate the host cell system in various respects.

Knowledge about effector contribution and their distinct role in pathogenesis of invasive NTS infections *in vivo* is rather scant. Therefore, we applied the neonate mouse model, which, in contrast to the commonly used streptomycin adult mouse infection model, allows the formation of SCVs inside infected enterocytes, followed by mucosal barrier penetration and subsequent bacterial dissemination without previous antibiotic depletion of the resident gut microbiota. In this study, we used this infection model to extend our knowledge on the role of individual SPI-2 effector proteins in establishment and progression of systemic *Salmonella* infections in the neonate host.

Oral infection of neonates with wildtype and ΔSPI-2 *Salmonella* resulted in similar bacterial loads of the gastrointestinal tract, but re-isolation rates of SPI-2-deficient mutants from systemic organs, such as liver and spleen, and expression of pro-inflammatory cytokines were significantly decreased. Interestingly, in contrast to the general understanding of SPI-2 as prerequisite for SCV formation *in vitro*, mutants were able to establish and maintain SCVs in neonatal intestinal enterocytes. In fact, SPI-2-deficient bacteria grow to high numbers inside SCVs, nonetheless, like in the adult host, are attenuated in virulence and hampered in disease progression. The establishment of an SCV is not only feasible in the absence of the SPI-2 effector protein subset, but also without the host adaptor protein myeloid differentiation primary response 88 (MYD88). The observation of enlarged SCV formation in the absence of SPI-2, however, is strictly limited to the neonatal period. This is due to the immature intestinal tract constituting a unique environment with pending epithelial renewal on the one hand,

whereas constant nutrient supply is ensured by fusion of the bacterial vacuole with transport vesides on the other hand. By evaluating isogenic single SPI-2 effector protein mutants, we detected that SPI-2 effectors enabling interaction with the host cell microtubule network and positioning of the SCV inside the host cell partly phenocopy the SPI-2 dependent phenotype *in vivo*. Results achieved in this study suggest that depletion of SPI-2 effectors involved in the interaction with the host cell transport machinery still allows bacterial replication in the neonate intestine. However, the absence of these effectors prevents SCV transmigration from the apical to the basolateral side of the enterocytes. Mutants are therefore "stuck" inside their SCVs, which ultimately diminishes systemic spread to liver and spleen and complete pathogenesis of *Salmonella in vivo*. Thereby, SPI-2 effector proteins are needless in terms of early steps in NTS pathogenesis in the neonate host, but become urgent for the pathogen in order to overcome the epithelial barrier and achieve systemic dissemination.

Zusammenfassung

Als einer der Haupterreger infektiöser Durchfallerkrankungen stellen nicht-typhöse Salmonellen (NTS), wie beispielweise *Salmonella enterica* subsp. *enterica* serovar Typhimurium, ein weltweites Gesundheitsproblem für Human- und Veterinärmedizin dar. Neben selbst-limitierender Gastroenteritis bei immunkompetenten Erwachsenen kann die Infektion von Neugeborenen mit diesem Erreger zu invasiven und potenziell lebensbedrohlichen Erkrankungen führen. Invasive Infektionen von Neugeborenen durch NTS treten vor allem in Entwicklungsländern mit geringen Hygienestandards und schlechter medizinischer Versorgung auf. Unter diesen Voraussetzungen kann der Pathogen häufig bei Fällen neonataler Sepsis und Meningitis isoliert werden. Die Pathogenität von *Salmonella* ist vor allem durch Virulenzfaktoren bedingt, die auf durch horizontalen Gentransfer erlangten chromosomalen Regionen, den sogenannten *Salmonella* Pathogenitäts Inseln (SPIs), kodiert sind. Die Effektorproteine von SPI-1 und SPI-2 werden dabei durch eigene Typ drei Sekretionssysteme (T3SS) in die Wirtszelle eingebracht. Die Funktion der SPI-kodierten Effektorproteine in der Wirt-Pathogen Interaktion wurde bereits in zahlreichen in vitro-Studien untersucht. SPI-2 Effektoren werden anhand dieser Studien als essenziell für den Aufbau eines besonderen intrazellulären Kompartiments, der „*Salmonella*-containing vacuole“ (SCV, engl. für *Salmonella*-beherbergende Vakuole) betrachtet, welche die bakterielle Replikation und das Überleben innerhalb der Wirtszelle ermöglicht. Ferner bewirken die translozierten Effektorproteine die Modulation und Manipulation verschiedener Wirtszellprozesse durch das Bakterium.

Das Wissen über die Beteiligung einzelner Effektoren in invasiven NTS Infektionen *in vivo* ist insgesamt, trotz zahlloser Studien, eher gering. Daher fand in dieser Studie das zuvor etablierte neonatale Mausmodell Anwendung. Im Gegensatz zum vielfach verwendeten adulten Streptomycin-Modell wird dabei die Bildung von SCVs in Enterozyten, gefolgt von der Penetration des Darmepithels und anschließender bakterieller Dissemination ohne vorherige Antibiotika Behandlung zur Verminderung der intestinalen Mikrobiota, ermöglicht. In der vorliegenden Arbeit wurde dieses Modell daher eingesetzt, um das Wissen über die Funktion einzelner SPI-2 Effektor Proteine in der Etablierung und im Krankheitsverlauf systemischer Salmonelleninfektionen im neugeborenen Wirt auszuweiten.

Während die orale Infektion von neugeborenen Mäusen mit Wildtyp und ΔSPI-2 Salmonellen hierbei zu ähnlicher bakterieller Belastung des Gastrointestinaltrakts führte, fielen sowohl die Re-Isolationsrate aus systemischen Organen wie Leber und Milz, als auch die Expression pro-inflammatorischer Zytokine im Falle der SPI-2-defizienten Mutante gegenüber dem Wildtyp deutlich geringer aus. Bemerkenswerterweise, und im Gegensatz zum weithin akzeptierten Dogma von SPI-2 als Voraussetzung für die Bildung von SCVs durch in vitro Daten, waren die SPI-2 defizienten

Bakterien in der Lage, SCVs in neonatalen intestinalen Enterozyten zu bilden und aufrecht zu erhalten. Tatsächlich wuchsen SPI-2 defiziente Bakterien innerhalb ihrer SCV zu einer großen Zellzahl heran, waren dabei jedoch, wie im adulten Wirt, avirulent und führten zu einem deutlich verminderten Krankheitsverlauf. Die Etablierung einer funktionalen SCV wurde nicht nur als unabhängig von SPI-2 Effektoren beobachtet, sondern kann darüber hinaus ohne das Wirtsadapterprotein „myloid differentiation primary response gene 88“ (MYD88) stattfinden. Die Beobachtung vergrößerter SCVs in Abwesenheit von SPI-2 ist dabei strikt auf die neonatale Periode limitiert. Dies gründet sich auf die Besonderheiten, die das intestinale Epithel in dieser Phase als Lebensraum bietet, da die kontinuierliche Erneuerung des Epithels noch aussteht, gleichzeitig aber die Fusion der bakteriellen Vakuole mit Transportvesikeln der Epithelzelle die intrazelluläre Nährstoffversorgung des Pathogens sicherzustellen scheint. Durch die Auswertung von isogenen Einzeleffektor Mutanten konnten wir zeigen, dass die SPI-2 Effektorproteine, welche die Interaktion mit dem Wirtszell-Mikrotubuli-Netzwerk, sowie die korrekte Positionierung der SCV innerhalb der Wirtszelle, ermöglichen, signifikant zum beobachteten SPI-2-abhängigen Phänotyp *in vivo* beitragen. Die Ergebnisse, die im Rahmen dieser Studie erzielt wurden, deuten darauf hin, dass die Abwesenheit von SPI-2 Effektoren, die an der Interaktion mit dem Transportsystem der Wirtszelle beteiligt sind, zwar weiterhin intrazelluläre Replikation, jedoch keine Transmigration der Enterozyten von apikal nach basolateral erlaubt. Die Mutanten sind in ihrer SCV innerhalb der Wirtszelle fixiert, wodurch die systemische Verbreitung der Bakterien verhindert und die weitere Pathogenese von *Salmonella* blockiert ist. Hierdurch konnten SPI-2 Effektor Proteine als abdingbar für frühe Schritte der NTS Pathogenese im neonatalen Darmepithel identifiziert werden. Sie sind jedoch für den Pathogen nötig, um die epitheliale Barriere zu überwinden und sich somit systemisch zu verbreiten.

1. Introduction

"Research cannot be forced very much. There is always danger of too much foliage and too little fruit." (Theobald Smith, discoverer of *Salmonella*)

1.1 General and theoretical background

1.1.1 Morphological and biochemical characteristics

The genus *Salmonella* comprises rod-shaped, Gram negative, facultative anaerobic bacteria within the family of *Enterobacteriaceae* named after the American veterinary surgeon and pathologist Daniel Elmer Salmon (Barlow and Hall 2002; Eng *et al.* 2015). First microscopic evidence succeeded in 1880 by Karl Eberth, who discovered the bacterium in the spleens of typhoid patients (Eberth 1880). Four years later, Georg Theodor Gaffky was the pioneer to successfully grow the pathogen in pure culture (Hardy 1999). With only rare exceptions, salmonellae are motile via peritrichous flagella and measure 2 to 3 by 0.4 to 0.6 µm in size (Drevets, Leenen, and Greenfield 2004).

Although considered a facultative intracellular pathogen, the lifestyle of *Salmonella* is non-fastidious and allows survival and even growth outside the living host under various environmental conditions (Jajere 2019). Salmonellae are highly resistant to different environmental stresses like temperature fluctuation, acidity, drought and limited nutrient availability (Fitzsimmons *et al.* 2018; Foster and Hall 1991; Spector and Kenyon 2012). For instance, the majority of serovars is viable and thrives at temperature ranges from 5-47 °C and optimal conditions between 32-35 °C (Pui *et al.* 2011). The undemanding nature of *Salmonella* strains also holds true regarding pH range and water requirement. Growth is preserved between pH values from 4 to 9, ensuring survival during different phases of the infectious process e.g. in the stomach or in dried foods in addition (Juven *et al.* 1984; Keerthirathne *et al.* 2016). Furthermore, salmonellae evolved strategies like cellular proton pumps, potassium and sodium antiport systems and acid tolerance responses, further increasing resilience under various circumstances and exposure to environmental stresses of different nature (Booth 1985; Foster and Hall 1990).

Versatility is additionally achieved by metabolism of a huge range of different substances. As chemoorganotrophs, salmonellae can utilize a huge variety of different substrates and nutrients by both respiratory and fermentative pathways (Ong *et al.* 2013). For most serotypes, this includes rapid reduction of nitrate to nitrite, fermentation of many different carbohydrates resulting in acid production, arginine, ornithine, dulcitol decarboxylate lysine, citrate and hydrogen sulfide utilization (Kim *et al.* 2013; Rivera-Chavez and Bäumler 2015; Warsi *et al.* 2019). In sum, these biochemical

features help provide high pathogenic potential to salmonellae with massive prevalence and clinical importance (Crump *et al.* 2015; Prager *et al.* 2000).

1.1.2 Taxonomy and nomenclature

Most *Salmonella* reference centers worldwide, including the Centers for Disease Control (CDC), adopted the nomenclatural system of salmonellae as recommended by the World Health Organization (WHO), although the nomenclature still remains disputable (Popoff, Bockemuhl, and Gheesling 2004). According to this nomenclatural system, the genus *Salmonella* consists of two broad species *Salmonella enterica* (*S. enterica*) and *Salmonella bongori* (*S. bongori*) based on 16S rRNA sequence analysis (Jajere 2019; Popoff, Bockemuhl, and Gheesling 2004). Further classification divides *S. enterica* into 7 subspecies (subsp.) and more than 2600 serovars/serotypes (ser.) to date (Table 1) (Gal-Mor 2019; Mezal *et al.* 2014). Subspecies are usually omitted in literature, e.g. *S. enterica* subsp. *enterica* serotype Typhimurium is normally shortened to *Salmonella* ser. Typhimurium or *S*. Typhimurium. In addition to the classification system of subspecies on a phylogenic level, serotyping is based on somatic (O), capsular (K), and flagella (H) antigens on the bacterial cell surface by means of the White-Kauffmann-Le Minor Scheme (Brenner *et al.* 2000; Issenhuth-Jeanjean *et al.* 2014). Most of the genes among different serovars are highly conserved. For instance, *S.* Typhi and *S.* Typhimurium, both infecting warm-blooded animals including humans, share 90 % of their total genes and only differ in their virulence factors, determining pathogenicity and host specificity as described later (McClelland *et al.* 2001; Sabbagh *et al.* 2010). However, each serovar or serotype harbors a unique combination of at least O and H antigens, thereby facilitating characterization and identification of isolates. In total, more than 50 % of serotypes described so far belong to *S. enterica* subsp. *enterica* I (Table 1) and account for approximately 99 % of *Salmonella* infections in humans and warm-blooded animals in sum (Guibourdenche *et al.* 2010; McClelland *et al.* 2001; Velge *et al.* 2012). In contrast, other subsp. of *S. enterica* and serovars of *S. bongori* are rarely found in humans or warm-blooded animals, but preferably colonize and infect cold-blooded species or are isolated from different environmental sources (Brenner *et al.* 2000).

Table 1: Classification of *Salmonella* species and the current number of serovars within each subspecies

Species belonging to the genus *Salmonella*, the assignment of the eight subspecies and particular number of serovars per subspecies to date (Achtman *et al.* 2012). In total, 2610 distinct serovars are discriminated currently.

genus	species	subspecies	no. of serovars
Salmonella	*S. enterica*	I *enterica*	1586
		II *salamae*	513
		IIIa *arizonae*	100
		IIIb *diarizonae*	341
		IV *houtenae*	73
		VI *indica*	13
		VII	
	S. bongori	V *bongori*	23

1.2 *Salmonella* - a pathogen of global health concern

1.2.1 Pathology of *Salmonella*

Salmonella is an important pathogen for both humans and animals worldwide, causing diseases of varying clinical manifestations. Typically, *S. enterica* subsp. *enterica* serovars are subdivided into two distinct groups, typhoidal and non-typhoidal (Gal-Mor, Boyle, and Grassl 2014). Furthermore, some serovars are host adapted, whereas others harbor features enabling infection of a variety of different hosts by the same serovar (Bäumler *et al.* 1998; den Bakker *et al.* 2011; Wheeler, Gardner, and Barquist 2018). For instance, typhoid fever is solely limited to humans and higher primates, whereas other warm-blooded vertebrate species are protected from infection by the causative agent *S.* Typhi and *S.* Paratyphi A and B (or uncommonly C), a pathogen eliciting the similar, but often less severe disease paratyphoid fever (Buckle, Walker, and Black 2012; Kingsley and Bäumler 2000).

Typhoid fever is usually acquired after ingestion of contaminated food or water (Crump and Mintz 2010). The acute illness is characterized by fever, headache, nausea, loss of appetite, constipation and sometimes diarrhea (Klotz *et al.* 1984). Symptoms are often non-specific and clinically non-distinguishable from other febrile illnesses and clinical severity is influenced by different factors, exacerbating documentation of both morbidity and mortality (Crump and Mintz 2010; Crump *et al.* 2015; Gibani *et al.* 2019). Symptoms of typhoid fever may vary from a mild illness with low grade fever, headache, fatigue, malaise, loss of appetite, cough, constipation and skin rash or rose spots (Klotz *et al.* 1984). In some fatal cases, severe complications including intestinal perforations, gastrointestinal hemorrhages, encephalitis and cranial neuritis can emerge, ultimately leading to potentially

life-threatening conditions and death in approximately 10 % of untreated and 1-2 % of hospitalized patients (Habte *et al.* 2018; Mogasale, Desai, *et al.* 2014). Typhoid occurs predominantly in low-income countries in association with poor sanitation, substandard hygiene, rather poor quality of life and lack of clean drinking water (Crump and Mintz 2010; Farooqui, Khan, and Kazmi 2009; Mogasale, Maskery, *et al.* 2014). According to the most recent estimates, between 11 and 21 million cases and 128 000 to 161 000 typhoid-related deaths occur annually worldwide, especially if typhoid or, in some cases, paratyphoid fever are left untreated (Crump, Luby, and Mintz 2004; Crump and Mintz 2010; Gibani, Britto, and Pollard 2018). Summarized, typhoid fever has been a human health problem for centuries and remains of global health concern to date.

On the other hand, *S.* Typhimurium, which is highly similar to *S.* Typhi on a genetic level, can infect multiple different warm-blooded hosts, including humans, cattle, pigs, sheep, horses, poultry and rodents (Edwards, Bruner, and Moran 1948; Hughes *et al.* 1971; Humphrey, Mead, and Rowe 1988; Sojka *et al.* 1977; Sojka *et al.* 1983; Sojka, Wray, and McLaren 1986; Wray, Sojka, and Bell 1981). Incidence of serovars in a certain species is not necessarily linked to virulence or pathogenicity. Two surveys in 1958 and 1966 investigated human clinical isolates and examined that two host adapted serotypes *S.* Dublin, primarily infecting cattle, and *S*. Choleraesuis, predominantly found in pigs, were only responsible for minor proportions of 0.6 % and 0.3 % of human Salmonellosis cases in England and Wales (Kingsley and Bäumler 2000). Despite this low incidence, both serovars are particularly invasive compared to others in men.

Non-typhoidal *Salmonella* (NTS) serovars, such as *S.* Typhimurium investigated in this study, are a global cause of usually self-limiting gastroenteritis. NTS clearly belong to the leading causes of foodborne illnesses worldwide with an estimation of 93.8 million annual cases, considering under-reporting and under-diagnosis, and 155 000 deaths each year due to complications resulting from gastrointestinal infections (Lestari *et al.* 2009; Majowicz *et al.* 2010). In recent years, studies on the incidence of global NTS infections revealed increasing incidence, with the majority of NTS burden found in the Southeast Asian and the Western Pacific regions (Majowicz *et al.* 2010; Prestinaci, Pezzotti, and Pantosti 2015). Gastrointestinal infections, similar to typhoid fever, generally stem from exposure to contaminated food or water and of the almost 94 million NTS cases reported annually, more than 80 million were estimated being of foodborne origin (McNamee and Smyth 2000; Tennant *et al.* 2016). Predominantly, infections emanate from pets or livestock and are the result of consumption of dairy products or meat of clinically inconspicuous, yet colonized animals, especially poultry and poultry-derived products like eggs (Foley *et al.* 1999; Hartmann *et al.* 1996; Petrie *et al.* 1977; Rice, Besser, and Hancock 1997; Wilcock, Armstrong, and Olander 1976). While the risk for *Salmonella* spp.-induced gastroenteritis is seen worldwide, incidence observed in places with reduced

sanitation and access to clean water is elevated. Thereby, NTS are of public health concern for human and veterinary medicine and lead to global economic losses in addition. Furthermore, they contribute to the economic burden of both industrialized and underdeveloped countries through the costs associated with surveillance, prevention and treatment of diseases (Crump, Luby, and Mintz 2004; Havelaar *et al.* 2015; Taitt *et al.* 2004). Besides self-limiting gastroenteritis, including onset of symptoms like diarrhea, vomiting, nausea, fever and abdominal pain within 6 to 72 hours following exposure, NTS can cause invasive diseases, dependent on e.g. age, gut flora and immune status of the host, as discussed later (Fluit 2005; Langridge, Wain, and Nair 2012; Strawn *et al.* 2014). However, gastroenteritis remains the most common manifestation of *Salmonella* infection worldwide, followed by bacteremia and enteric fever (Majowicz *et al.* 2010). Among NTS serovars, *S.* Typhimurium, *S.* Enteritidis, *S.* Heidelberg and *S.* Newport are of particular importance in an epidemiological regard (Eguale 2018; Galanis *et al.* 2006).

1.2.2 Transmission

From an evolutionary point of view, the success of bacterial pathogens is dependent on their ability to colonize and cause disease in susceptible hosts. *Salmonella* is ubiquitous and, as explained previously (1.1.1), highly persistent even in hostile and dry environments, where it can survive for periods from days up to several months (Davies and Wray 1996; Finn *et al.* 2013). Furthermore, especially serovars of *S. enterica* are broadly distributed among different host species, including diverse mammals and birds (Rabsch *et al.* 2002). Certain hosts can serve as reservoirs and, with only rare exceptions of host-restricted serovars like *S.* Typhi, the vast majority of *S. enterica* serovars displays a broad host range for infection and colonization in equal measure (Bäumler *et al.* 1998). In comparison to other pathogens, salmonellae are widely distributed in animals and the environment, exhibit a high tenacity and persistence and inhabit diverse biological niches (Waldner *et al.* 2012). Equally important as survival outside the living host even under harsh conditions is how effectively these pathogens are transmitted between individual hosts. The primary route of human infections is by the consumption of meat or animal products contaminated via contact with animal feces (Hornick *et al*. 1970). Hereby, butchering of clinically inapparent, yet colonized animals might lead on to entry into the grocery chain (Lawley *et al*. 2008). Consequently, an overwhelming amount of 94 % of *Salmonella* infections is estimated to be of foodborne origin and less common waterborne, via contact to farm or pet animals or environmental transmission (Hale *et al.* 2012; Hoelzer, Moreno Switt, and Wiedmann 2011; Pees *et al.* 2013). Furthermore, salmonellae have been detected in animal herds, e.g. of pigs and cattle, with animal-to-animal transmission in a serovar dependent manner (Hendriksen *et al.* 2004).

NTS may have a broad host spectrum and are frequently zoonotic (Gordon 2011). A prominent example of a non-typhoidal, non-human-restricted or adapted salmonellae and perhaps the

best-studied is *S. enterica* subsp. *enterica* ser. Typhimurium (STy) (Finlay and Brumell 2000; Tsolis *et al.* 2011). In various studies, it has been investigated in infections of different cell types and animal models. Like other salmonellae, it is most probably acquired by food poisoning after oral ingestion of improperly cooked meat, raw milk, eggs or other dairy products or contaminated fruits and vegetables after manuring with animal feces and only to a minor extent via drinking or bath water (Wang *et al.* 2008). The infectious dose varies dependent on host species, age and immune status (Byrd *et al.* 1998; Correia-Gomes *et al.* 2014; Fedorka-Cray *et al.* 1994). After oral uptake, infection with NTS like STy and other *S. enterica* subsp. *enterica* serovars as well, usually results in self-limiting gastroenteritis as global key players in infectious diarrheal diseases (Mead *et al.* 1999; Santos *et al.* 2009; Tauxe 1997). Furthermore, extra-intestinal and more invasive infections are also frequent in susceptible hosts, predominantly in sub-Saharan Africa, where NTS are associated with HIV in adults and malnutrition, anemia, malaria and HIV in children (Biggs *et al.* 2014; Feasey *et al.* 2015; Graham *et al.* 2000).

1.2.3 Virulence factors and evolution

Serovars within *S. enterica* subsp. *enterica* are closely related as shown by analysis of orthologous genes. The divergence in the nucleotide sequence ranges between 3.8 and 4.6 % and differences in deduced amino acid sequences between 0.7 and 1.3 % (Bäumler *et al.* 1998; Sabbagh *et al.* 2010; Wang, Huang, and Huo 2014). Genetic variation of different *S.* Typhi isolates from all over the world was extremely low, revealing a highly conserved and clonal relation among isolates and suggesting that they emerged from a single progenitor (Baker and Dougan 2007; Holt *et al.* 2008). Clonality is a characteristic feature of human-restricted pathogens (Achtman 2008). STy is capable of causing an invasive and systemic typhoid-like disease in susceptible mice and this murine model served in unraveling *Salmonella* genes involved in pathogenicity (Kaiser *et al.* 2012). In total, more than 100 different genes have been implicated as contributors of *Salmonella* virulence. Of those, many are shared between both broad host range *S.* Typhimurium and other non-typhoidal *S. enterica* serovars and human restricted *S.* Typhi (Andrews-Polymenis *et al.* 2010; Bäumler *et al.* 1998; Sabbagh *et al.* 2010; Tanner and Kingsley 2018).

Certain genetic traits, however, distinguish *S. enterica* serotypes from *S. bongori* or other related *Enterobacteriaceae* like *Escherichia coli* (*E. coli*) or *Shigella* spp. (Langridge *et al.* 2015; Winfield and Groisman 2004). As holds true for very many other pathogens, successful colonization, invasion and infection of the host by salmonellae requires the expression and secretion of particular virulence factors. These factors enable distinct steps in pathogenesis and survival under various conditions inside the living host (Coleman and Haller 2018; Ilyas, Tsai, and Coombes 2017). Acquisition of genes in general, e.g. by horizontal transfer, can confer new phenotypes to the recipient bacteria, thereby offering chances of adaptive changes that maximize fitness in a given niche and increase pathogenicity

or virulence in a global manner (Groisman and Ochman 1996; Ochman, Lawrence, and Groisman 2000). The genome of STy, for instance, was modeled by various gene acquisition events and accrued DNA changes that have undergone selection by regulatory evolution, allowing survival in diverse niches via regulatory circuitry of gene expression (Bäumler 1997; Groisman and Ochman 1997).

Virulence evolution of *Salmonella* after divergence from the closely related species *E. coli* by a speciation event from a common ancestor was proposed to have occurred in at least 3 stages between approximately 140 and 25 million years ago (Bäumler *et al.* 1998; Ehrbar and Hardt 2005; Winfield and Groisman 2004). Evolutionary steps are defined by the acquisition of certain genes and virulence traits expanding feasible niches to colonizing and infecting animals. Pathogenicity islands (PIs) are gene clusters encoding virulence factors incorporated in the genome of different bacteria acquired by horizontal gene transfer (Gal-Mor and Finlay 2006). In case of *Salmonella* genomics, 21 multi-gene loci linked to infection, called *Salmonella* Pathogenicity Islands (SPIs), have been identified mostly in both species and across all subspecies (McClelland *et al.* 2001). However, PIs are unequally critical in terms of pathogenesis and pathogenicity and genetic flux with the *Salmonella* species and subspecies sum up to define host range as well as disease phenotype (Figure 1) (Bäumler 1997; Fookes *et al.* 2011).

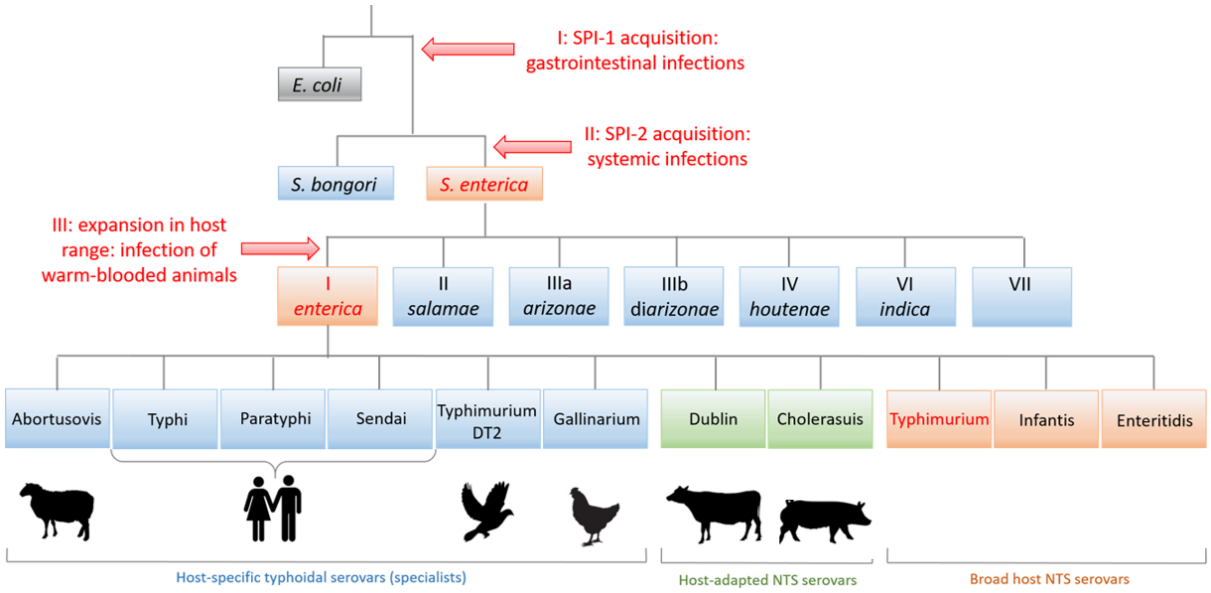

Figure 1: Evolutionary history, phylogeny and host specificity of *Salmonella* spp. and serotypes

Salmonella diverged from a common ancestor shared with *Escherichia coli* (*E. coli*) by development of *Salmonella* pathogenicity island-1 (SPI-1). Via horizontal gene transfer, a new niche could be colonized and gastrointestinal infections of cold-blooded animals became feasible. SPI-2 harbored by *S. enterica*, but not *S. bongori* serovars, enables systemic dissemination of the pathogen inside the living host. This feature was accomplished approximately 35 million years ago. The most eminent serovars for human and veterinary medicine belong to subspecies I of *S. enterica*. In the third phase of *Salmonella* evolution, the host range was expanded to allow infection of warm-blooded animals including humans by serovars of *S. enterica* subsp. *enterica*. While *S. bongori* and *S. enterica* subspecies II, IIIa, IIIb, IV, VI, and VII are mainly associated with cold-blooded vertebrates, members of *S. enterica* subspecies I are

most frequently isolated from avian and mammalian hosts . According to their host range and elicited disease, serovars of *S. enterica* subsp. *enterica* are clustered into host specific and broad host range, as well as typhoidal and non-typhoidal subgroups. In this and many other studies, *S.* Typhimurium (labeled in red) is used for infection of susceptible mice, causing a typhoid-like disease and potentially life-threatening conditions (modified from (Aleksic, Heinzerling, and Bockemuhl 1996; Bäumler *et al.* 1998; Desai *et al.* 2013; Gal-Mor 2019).

Virulence factors encoded within different SPIs contribute to the whole genome of *Salmonella* to a comparably high amount with approximately 4 % (Bowe *et al.* 1998; Dos Santos, Ferrari, and Conte-Junior 2019). As the first and initial step in virulence evolution, *Salmonella* pathogenicity island-1 (SPI-1) was obtained by plasmid- or phage-mediated horizontal gene transfer (Bäumler *et al.* 1998). SPI-1 was likely acquired by a common ancestral to all *Salmonella* serotypes early in evolutionary divergence, since it is present in all phylogenetic lineages of the genus *Salmonella,* but absent from closely related bacteria like *E. coli* (Mills, Bajaj, and Lee 1995). Effector proteins encoded by SPI-1 were described to mediate mechanisms used by *Salmonella* during the intestinal phase of infection in previous studies. This includes invasion of intestinal epithelial cells both *in vivo* and *in vitro*, thereby enabling an early key step in *Salmonella* pathogenesis (Galan and Curtiss 1989; Watson *et al.* 1995; Zhang *et al.* 2014). In accordance to this, attainment of SPI-1 enabled intestinal infections as a new potential reservoir for *Salmonella* (Bäumler *et al.* 1997). In a second evolutionary key step, two species emerged within the genus *Salmonella,* distinguishable by presence (*S. enterica)* or absence (*S. bongori*) of a second virulence determinant SPI-2. Divergence of their lineages was accompanied by an impact on disease potential, since the key feature enabled by possession of SPI-2 virulence factors is enabling invasion of deeper body tissue and systemic dissemination of *S. enterica* subspecies (Hensel *et al.* 1997; Ochman and Groisman 1996). While *S. bongori* and *S. enterica* subspecies II, IIIa, IIIb, IV, VI, and VII are predominantly isolated from cold-blooded animals like e.g. reptiles, serovars of *S. enterica* subsp. I are capable of infecting a large range of different warm-blooded animals and are frequently detected as colonizers or pathogens of the intestinal tract of mammals and birds (Bäumler and Fang 2013). This expansion in host range is considered the third step in evolution of *Salmonella* virulence (Figure 1) (Roumagnac *et al.* 2006).

1.3 Pathogenesis of non-typhoidal *Salmonella* (NTS)

To date, differences in the genome of distinct *S. enterica* subsp. *enterica* serovars, like *S.* Typhi and *S.* Typhimurium, is of particular research interest in various studies, aiming in unraveling possible causes for different forms of disease caused by individual serotypes (Bäumler and Fang 2013; Gal-Mor, Boyle, and Grassl 2014; Sabbagh *et al.* 2010). However, major steps in infection and disease progression of invasive non-typhoidal *Salmonella* (iNTS) infections and typhoid fever are identical. Infection of mice with *S.* Typhimurium, usually causing gastroenteritis in humans, resembles systemic

dissemination observed in typhoid fever patients (Tsolis, Kingsley, *et al.* 1999). An infection is presumably acquired by ingestion of contaminated food (Gomez *et al.* 1997). Certain groups are at an increased risk of suffering from iNTS infections, including children under the age of 5, elderly and immunosuppressed people (Eng *et al.* 2015). In these risk groups, chances of the pathogen penetrating the epithelial cell lining of the intestinal tract after entering the digestive tract are elevated, which might lead to life-threatening conditions, including bacteremia, meningitis and osteomyelitis (Church *et al*. 1998; Yang *et al*. 2002). Infections of young children display significantly higher morbidity and mortality than observed in adults.

Salmonellae initiate their own engulfment into non-phagocytic enterocytes in the small intestine via SPI-1 effector proteins (Hansen-Wester, Stecher, and Hensel 2002). Activation of signal transduction pathways by bacterial SPI-1 virulence factors induces reorganization of the actin cytoskeleton of the host cell, leading to membrane ruffling and absorption of the pathogen (Monack, Mueller, and Falkow 2004; Takaya *et al.* 2003). After crossing the epithelial barrier, *Salmonella* gains access to the underlying *lamina propria* and residing immune cells such as neutrophils and macrophages (Vazquez-Torres *et al.* 1999). Invasion, survival and even replication in these host cells facilitates systemic dissemination to the liver, spleen, bone marrow and gallbladder (Johnson, Mylona, and Frankel 2018; Mastroeni *et al.* 2003). Furthermore, *Salmonella* persists inside host cells as a crucial feature of pathogenesis (Bakowski, Braun, and Brumell 2008). This persistence, immune evasion and intracellular survival inside the host cell is accomplished via SPI-2 effector proteins, thereby conferring NTS pathogenesis, as discussed later (1.4).

Different reasons render infants and toddlers highly susceptible to iNTS infections. The gastrointestinal tract of men and mice is rather immature at birth (Birchenough *et al.* 2017; Walthall *et al.* 2005). The gut barrier shields the sterile body interior from the environment, thereby providing the first line of defense to iNTS and mucosal resistance of the gut epithelium to NTS is a complex and multifactorial system (Houghteling and Walker 2015; Macierzanka *et al.* 2014; Viggiano *et al.* 2015; Zhang, Hornef, and Dupont 2015). Cellular and humoral arms of immunity, the gut epithelial layer as well as the gut microbiota have been discussed to contribute to the so-called "colonization resistance", which confers a comparably high degree of resistibility to *Salmonella* infections in healthy, immunocompetent, adult individuals (MacLennan *et al.* 2008; Nyirenda *et al.* 2014; Vazquez-Torres *et al.* 1999; Veereman-Wauters 1996). However, the protective mucus layer in the gut as well as the residing microbiota are developed postnatally (Rakoff-Nahoum *et al.* 2015; Schroeder 2019). In contrast to other studies employing the streptomycin mouse model to study *Salmonella* infections, oral infection of neonate mice harboring an immature microbiome is conducted without any antibiotic treatment prior to infection (Kaiser *et al.* 2012; Zhang *et al.* 2014). Furthermore, maturation of the intestinal tract and

development of barrier integrity also involves cellular changes (Kelly, Begbie, and King 1992). Microfold (M) cells, constituting a primary entry port for *Salmonella,* start to mature after birth in mice (Clark *et al*. 1996; Jepson and Clark 2001; Jones, Ghori, and Falkow 1994). This forces the pathogen to switch to an alternative invasion strategy in the murine neonatal small intestine via SPI-1-induced uptake by epithelial cells, since M cells are not fully developed at an early age (Zhang *et al*. 2014). Additionally, enterocytes of the gut epithelium undergo constant and rapid renewal in later life via proliferation of stem cells, e.g. located in the intestinal crypts (Renz, Brandtzaeg, and Hornef 2012). Thereby, barrier integrity of the gut epithelium is maintained and NTS infections in adults usually result in self-limiting gastroenteritis instead of severe, disseminative infections (Peterson and Artis 2014). To a large extent, this is due to continuous desquamation of intestinal enterocytes every 3 to 5 days, accompanied by the loss of the intracellular pathogen (Eastwood 1977; Gordon and Hermiston 1994). In mice, the villus-crypt architecture of the gut, accompanied by constant epithelial cell renewal, is developed between 10 and 12 days after birth, offering chances of prolonged intraepithelial proliferation of *Salmonella* in the murine neonate intestine. As compared to adults, newborns possess a premature adaptive immune system. Invasion of the pathogen induces cytokine expression after e.g. recognition of structures on the bacterial surface and sensing by pattern recognition receptors (PRRs), promoting immune cell infiltration in both adults and infants. Nevertheless, chances for the neonate to die from the massive gut inflammation and pathogen dissemination before an adaptive immune response can be mounted to control the infection are substantially higher than in immunocompetent, healthy adults (Bermudez-Brito *et al.* 2012; Hornef and Fulde 2014; Machado *et al.* 2014). In this context, failure in an appropriate T cell maturation in HIV patients and deficiencies in the adaptive immune system were attributed to an increased probability of NTS to disseminate systemically and cause bacteremia and other life-threatening consequences (Morpeth, Ramadhani, and Crump 2009; Preziosi *et al.* 2012). This immaturity of the host, especially the gut and the immune system, augments vulnerability to NTS infections in the neonate.

1.4 *Salmonella* pathogenicity island-2 (SPI-2)

1.4.1 Genetics

In contrast to other SPIs, SPI-1 and SPI-2 encode an own type three secretion system (T3SS) besides their respective effector proteins and are hence considered the major virulence determinants of *S. enterica* serovars (Ibarra and Steele-Mortimer 2009). T3SSs are complex, syringe-like translocation machineries found in different, primarily pathogenic, bacteria like *Yersinia* and *Shigella* species and in association with a translocon pore that is formed in the target host membrane (Figure 2) (Coburn, Sekirov, and Finlay 2007; Mueller, Broz, and Cornelis 2008). In total, more than 20 different protein

compartments are assembled to form a needle-like injection apparatus, facilitating direct insertion of effector proteins into the host cell cytosol (Haraga, Ohlson, and Miller 2008). Some of the proteins within the T3SS are homologous to those involved in assembly of flagella, suggesting an evolutionary relation (Kubori *et al.* 1998; Marcus *et al.* 2000).

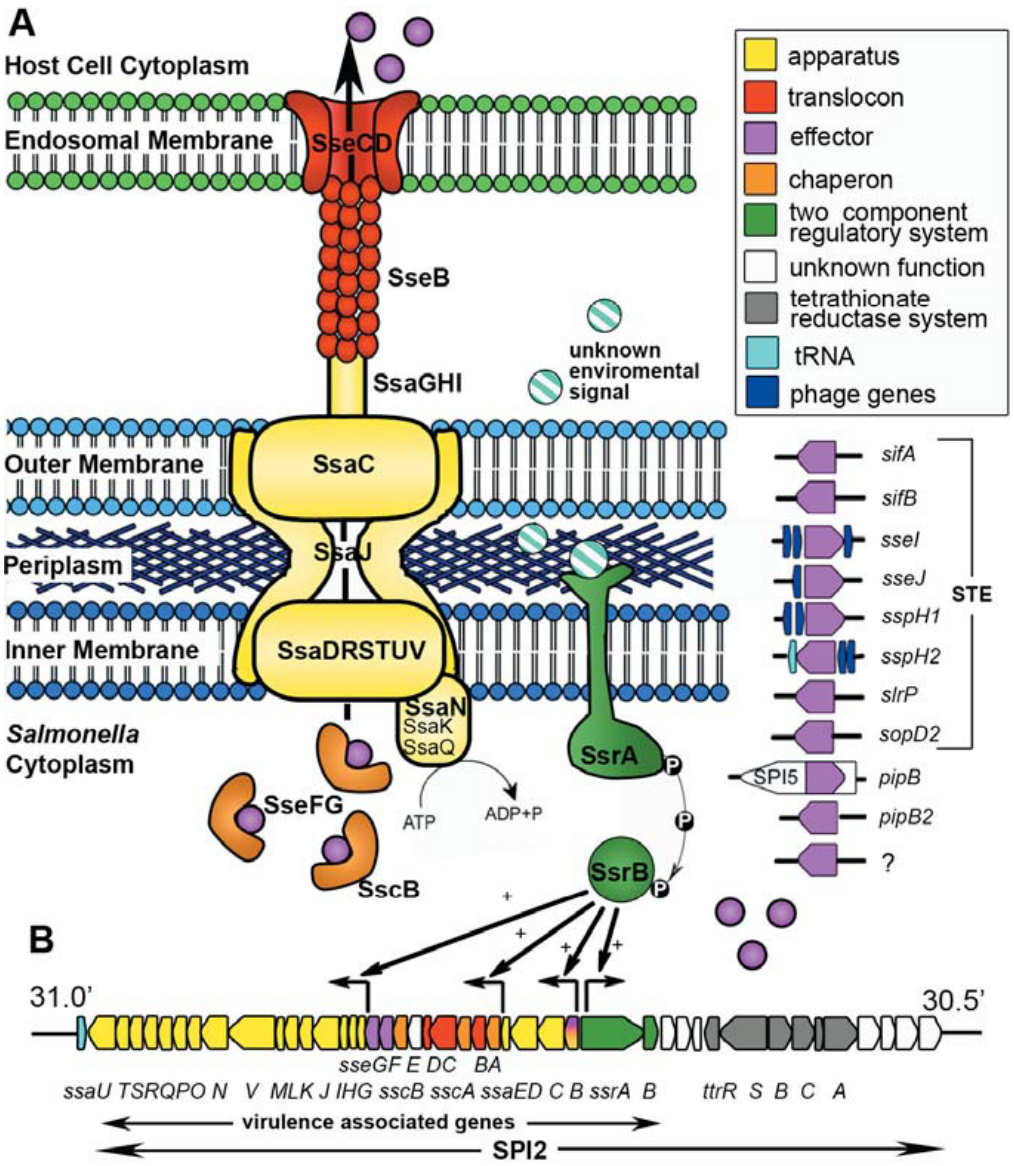

Figure 2: Schematic representation of the T3SS and genetic structure of SPI-2

The subcellular localization of the multi component structure forming the T3SS to translocate effector proteins of SPI-2 into host cells (A) and the genetic structure of the pathogenicity island (B) are depicted. The needle-like injection machinery spans the inner and outer membrane of the bacterial envelope (shown in yellow). The translocon consisting of SseBCD (red) forms a pore in the host cell endosomal membrane and facilitates translocation of effector proteins (violet) directly into the host cell cytosol. In addition to little virulence factors encoded within SPI-2 itself, a remarkable proportion of effectors translocated by the T3SS of SPI-2 is encoded on separate loci. Some effector proteins rely on their respective chaperone (orange) for activation and functionality. Expression of SPI-2 genes and other effectors is regulated by the SsrAB two component regulatory system (green), although the precise mechanism underlying regulation is not thoroughly understood. Copied from (Kuhle and Hensel 2004).

SPI-2 is located at 30 centisomes of the *S. enterica* chromosome and of crucial importance after host cell invasion as a requirement for intracellular replication in cultured epithelial cells and survival in macrophages (Deiwick *et al.* 1998; Hapfelmeier *et al.* 2005; Ochman *et al.* 1996). Like other horizontally acquired genes, SPI-2 shows a substantially higher A+T content than ancestral *Salmonella* DNA (Fass and Groisman 2009; Shea *et al.* 1996). It consists of more than 40 different genes, 28 of which are secreted effector proteins with different functions and 15 protein subunits to build the T3SS (Coombes *et al.* 2004; Figueira and Holden 2012). The effectors have diverse purposes in terms of host cell modulation and manipulation and can be clustered into different functional subgroups in accordance. However, certain effectors participate in different processes and comprise further biochemical activities (Figueira and Holden 2012). Moreover, distinct effectors share functional capacities and generate protein redundancies in terms of certain processes. In some cases, especially in complex processes within the host cell, an interplay of effects of various proteins is essential for functionality (Hensel, Nikolaus, and Egelseer 1999). In summary, SPI-2 effector protein-mediated host cell modulation is a complex machinery with fundamental repercussions on virulence and NTS pathogenesis.

1.4.2 Function and regulation

As mentioned previously, various host cell processes can be interfered or modulated by SPI-2 effector proteins. In general, SPI-2-deficient *Salmonella* are attenuated in virulence in adult mouse infections and diminished in intracellular survival in cultured macrophages as well as replication in epithelial cells, since SPI-2 effectors facilitate manipulation of vesicular trafficking of the host cell (Cheminay, Mohlenbrink, and Hensel 2005; Leung and Finlay 1991). Once internalized into the intestinal epithelium after oral uptake, STy subverts the physiological maturation of its vacuolar compartment and its acidic pH to form a specialized intracellular compartment, known as the *Salmonella*-containing vacuole (SCV) (Kolodziejek and Miller 2015; Steele-Mortimer 2008).

Sustained progress has been made in revealing mechanisms and molecular triggers that lead to induction of SPI-2 gene expression (Arpaia *et al.* 2011). In this context, multiple signals have been implicated in induction of SPI-2 expression *in vitro*, e.g. cation deprivation, low pH and phosphate starvation (Cirillo *et al.* 1998; Deiwick *et al.* 1999; Rappl, Deiwick, and Hensel 2003). Nevertheless, the physiological cues responsible for induction of the SPI-2 T3SS in the phagosome and the degree of their necessity remain a matter of debate. SpiR/SsrB is a SPI-2 encoded two-component system, which is essential for induction of the T3SS-2 and all effectors within the PI, as well as those encoded outside the virulence locus (Worley, Ching, and Heffron 2000). Its expression is controlled by the ancestral two-component systems OmpR/EnvZ and PhoP/PhoQ (Garmendia *et al.* 2003; Heithoff *et al.* 1999; Miller, Kukral, and Mekalanos 1989). In general, microorganisms invade host cells or are ingested by

phagocytes like e.g. macrophages and are trapped in a membrane-bound compartment or phagosome. Sequential fusion events with a series of endomembrane compartments result in maturation and a declined luminal pH, which confers microbicidal properties to the phagosome (Harrison *et al.* 2003). This maturation process is subverted by *Salmonella* and the pathogen evades the hostile environment of the phagosome via its effector proteins (Cuellar-Mata *et al.* 2002). As a specialized, membrane-bound compartment, the SCV provides protection from the host cell's immune system. It allows modulations of vesicular trafficking to bypass lysosomal degradation, enabling intracellular survival, persistence and replication of the pathogen through the action of distinct virulence factors (Brumell *et al.* 2002; D'Costa *et al.* 2015; Garcia-del Portillo and Finlay 1995; McGourty *et al.* 2012). Basically, the SCV is a modified phagosome with low pH and this is exploited as an environmental cue to alter the pathogen's transcription profile (Valdivia and Falkow 1997). Hence, following invasion, adaptive responses of the PhoP/PhoQ system harbored by STy sense the phagosomal milieu within the bacterial vacuole, such as low pH and magnesium ion (Mg^{2+}) concentration, leading to the expression of virulence determinants of SPI-2 (Dalebroux and Miller 2014). This turns the phagosome into a replicative niche, the SCV, with virulence factors of SPI-2 transported from the inside of the vacuolar compartment across the SCV membrane into the host cell cytosol, where the effector proteins operate (Marcus *et al.* 2000). During disease progression, the pathogen experiences low pH e.g. in the phagosomal vacuoles of epithelial cells and macrophages in the gut. This is enabled via the PhoP/PhoQ two-component regulatory system, which is also important for survival in the presence of reactive oxygen (ROS) or nitrogen species. Hence, null mutations in these genes severely attenuates STy virulence in mice infections (Miller, Kukral, and Mekalanos 1989). In addition to constituting an immune evasion strategy, virulence factors endowing the intracellular pathogen with vesicular trafficking modulation was discussed as an approach to ensure nutrition inside the host cell (Liss *et al.* 2017). Besides this, expression of SPI-2 genes in the gut was suggested to contribute to intracellular transit from the apical to the basolateral side of polarized epithelial cells in the gut (Brown *et al.* 2005; Muller *et al.* 2012).

1.4.3 Host factors influencing pathogenesis of *Salmonella*

Pathogens have evolved mechanisms to antagonize the host's innate immune system in various ways, since it is of special importance in limiting microbial replication and pathogen spread before an adaptive immune response can be established (Woolhouse *et al.* 2002). In this context, PRRs sense detection of conserved pathogen-associated molecular patterns (PAMPs) on the surface of bacteria and hence are crucial for the innate immune system (Medzhitov 2007). Expression of toll-like receptors (TLRs) as important members of PRRs, allows recognition of different microbial ligands e.g. by enterocytes as well as innate immune cells. Thereby, TLRs link detection to the induction of

mechanisms to defeat invading pathogens via production of reactive oxygen and nitrogen species, expression of antimicrobial peptides and maturation of dendritic cells (DCs) (Akira, Takeda, and Kaisho 2001; Iwasaki and Medzhitov 2004). Thus, TLRs significantly contribute to the earliest steps of the host response to an infection, but besides antimicrobial sensing, TLR activation can also induce non-transcriptional effects, including rapid acidification and maturation of the phagosome (Arpaia *et al.* 2011; Sivick *et al.* 2014). This acidification was discussed as an inducer for SPI-2 gene expression in STy infections, after the ligands of the pathogen have been recognized by mainly TLR2 (lipoproteins), TLR4 (lipopolysaccharide, LPS), and TLR5 (flagellin) (Fulde *et al.* 2018; Hapfelmeier *et al.* 2005; Kawai and Akira 2005; O'Brien *et al.* 1980; Royle *et al.* 2003). Failure to rapidly acidify the endosomal compartment in TLR-deficient mice led to a striking replication and SCV maintenance deficiency in bone marrow macrophages (BMMs) *in vitro,* due to abrogated induction of SPI-2 expression in the absence of TLR2, 4 and 9 (Arpaia *et al.* 2011). However, TLR-dependent SPI-2 induction was only dispensable in the systemic phase of adult STy infections. After oral administration, SPI-2 effectors were required to achieve virulence and systemic spread even in TLR-deficient animals (Sivick *et al.* 2014).

The two most prevalent mouse strains BALB/c and C57BL/6 share the feature of a point mutation within the *Slc11a1* gene, which encodes the natural resistance associated macrophage protein 1 (NRAMP1) (Gruenheid *et al.* 1997; Govoni *et al.* 1996; Vidal *et al.* 1996). This increases the susceptibility to different intracellular pathogens, like *Leishmania*, *Mycobacterium* and *Salmonella* (Araujo *et al.* 1998; Hackam *et al.* 1998). The function and mechanism of this protein remain incompletely understood, but it is known that *Nramp* is expressed exclusively in professional phagocytes and encodes an integral membrane protein that shares structural characteristics with ion channels and transporters of e g. iron and manganese (Forbes and Gros 2003). The removal of ions from the SCV by NRAMP1 might predict bacterial replication, increasing resilience of macrophages with a functional *Slc11a1* allele isolated from 129sv mice (Cuellar-Mata *et al.* 2002).

1.4.4 SPI-2 dependent intracellular lifestyle *in vitro* and *in vivo*

Infection and pathogenesis are multicomponent processes, involving invasion, proliferation, replication, cell evasion and dissemination at distinct body sites. To a large extent, these intermediate steps are facilitated by expression of virulence factors encoded by SPI-2 as virulence determinants in the case of *Salmonella* in susceptible mice. Pathogenesis of *Salmonella* strongly depends on the ability to invade and replicate within host cells during early phases of the infection, prior to breaching the epithelial gut barrier and during systemic dissemination (Shea *et al.* 1999). In epithelial cells, STy exhibits a bimodal lifestyle, either proliferating in its established SCV as an intracellular replicative niche or freely in the cytosol (Brumell, Rosenberger, *et al.* 2001; Steele-Mortimer *et al.* 2002).

Self-induced or host-mediated lysis of the SCV, however, constitutes a double-edged sword for the pathogen. On the one hand, hyper replication in the host cell cytosol is a comparably rare phenomenon observed in less than 20 % of epithelial cells investigated via a semi-quantitative single-cell analysis (Malik-Kale, Winfree, and Steele-Mortimer 2012). In contrast to vacuolar bacteria, in which the SPI-2 T3SS is induced, cytosolic bacteria have been shown to be transcriptionally different and SPI-1-T3SS-induced, although being situated in an intracellular state (Knodler *et al.* 2010). Hyper-replication in the host cell cytosol might eventually result in pyroptosis and epithelial cell death marked by loss of the plasma membrane integrity. Following cell lysis, pro-inflammatory cytokines are released by activating caspase 1 and by induction of caspase 3/7, bacteria gain access to the extracellular space, which might constitute a strategy for further dissemination of invasion-primed bacteria (Knodler *et al.* 2010; Knodler 2015). Several *in vitro* studies with non-polarized epithelial cells have confirmed cytosolic replication of *Salmonella* contributing to the net growth of the pathogen only to a minor extent compared to vacuolar replication, that unequivocally relies on a functional SPI-2 T3SS (Malik-Kale, Winfree, and Steele-Mortimer 2012). Hyper-replication is a phenomenon solely investigated in cultured epithelial cells, but not phagocytes like macrophages so far and single bacteria rather than "clumps" invade host cells *in vivo* (Grant *et al.* 2012; Knodler *et al.* 2010). However, SPI-2 effectors are undeniable crucial when it comes to macrophage survival after entering the *lamina propria* in the gut and hence, SPI-2-independent replication possibly occurring in epithelial cells of the intestinal lining probably plays an inferior role in overall STy pathogenesis (Cirillo *et al.* 1998; Hensel *et al.* 1998; Fields *et al.* 1986). Considering this, possession of SPI-2 effectors is regarded as a crucial feature for intracellular replication and disease progression of STy, although cytosolic replication works irrespective of SPI-2 effector proteins (Chakravortty, Hansen-Wester, and Hensel 2002; Garai, Marathe, and Chakravortty 2011; Shea *et al.* 1996)

Investigations of SPI-2-deficient *Salmonella* in the streptomycin mouse model already unraveled a necessity of SPI-2 effector proteins for development of full systemic virulence in adult infection experiments (Browne *et al.* 2008). While SPI-1 effectors are required for invasion of non-phagocytic epithelial cells, primarily limiting their requirement to breaching the intestinal barrier, SPI-2 becomes essential in the post-invasive phase for disease progression in typhoid and enteric fever as well as gastroenteritis caused by *Salmonella* (Malik-Kale, Winfree, and Steele-Mortimer 2012; Marlovits and Stebbins 2010). In line with this, SPI-2 mutants in contrast to ΔSPI-1 STy, remain attenuated when the intestinal barrier is circumvented by intraperitoneal (i.p.) or intravenous (i.v.) injection. This has been suggested to be attributable to the replication deficiency observed in *in vitro* studies plus an elevated vulnerability to killing by macrophages in addition (Fields *et al.* 1986). These synergistic effects in terms of decreased virulence in SPI-2-deficient *Salmonella* trace back to the same origin. Effector proteins that are translocated through the SCV membrane into the host cell cytosol facilitate conversion of the

early vacuolar compartment into a replicative niche (1.4.2). Although lysosomal fusion is avoided through the action of certain effector proteins, SCVs are still associated with lysosomal markers like e.g. LAMP-1 and this association typically precedes replication (Meresse *et al.* 1999; Steele-Mortimer *et al.* 1999). On the one hand, effectors function in immune evasion and avoidance of lysosomal degradation (Lei *et al.* 2016). Effector proteins modulating endosomal trafficking responsible for prevention of fusion with lysosomes also ensure intracellular localization of the SCV in a juxtanuclear position. Furthermore, they might contribute to intracellular nutrition by ensuring continuous exchange of the SCV with host cell endosomes as a prerequisite for replication (Liss *et al.* 2017; Noster *et al.* 2019; Popp *et al.* 2015). Colitis caused by either SPI-1 or SPI-2-deficient STy was less pronounced in comparison to a wt STy infection in the streptomycin mouse model, but certain cytokine and interleukin expression as mediators of innate immune responses and inflammation could still be examined in the absence of decisive virulence proteins of either SPI-1 or SPI-2 (Hapfelmeier *et al.* 2005). This confirms that a combination of both efficient invasion and transfer across the epithelial gut barrier, as well as evasion from the immune system, are required for functional systemic dissemination, replication and disease progression and highlights the role of SPI-1 and SPI-2 for virulence in the mouse model. This is further emphasized by various studies stating SPI-2 to be a prerequisite for efficient intracellular replication inside vacuoles in tissue culture experiments and the adult mouse model (Galan 2001; Shea *et al.* 1996; Waterman and Holden 2003).

Intracellular replication inside the SCV is a characteristic feature of *S. enterica* to rapidly enhance net growth. This process requests proper nutrition as a feature of high importance for the pathogen, which is also mediated via SPI-2 effectors by formation of tubular extensions known as *Salmonella*-induced filaments (SIFs) (Birmingham *et al.* 2005; Brumell, Tang, *et al.* 2001; Liss *et al.* 2017). Many auxotrophs were shown to be attenuated in terms of virulence in adult mice and replication deficiencies were examined for mutants depleted in tryptophan, tyrosine, phenylalanine, purines and pyrimidine synthesis (Chaudhuri *et al.* 2009; Thorne and Corwin 1975).

In total, the typical isolation of *Salmonella* from lymphatic tissue and secondary lymphoid organs, such as the mesenteric lymph node, described in previous studies uniformly indicates that survival of the pathogen within macrophages is a critical feature to achieve systemic dissemination and invasive infections. Expression and translocation of effector proteins of SPI-2, furthermore, was stated to be important to manage intravacuolar replication via SCV formation and maintenance e.g. in HeLa cell cultures and resistance to macrophage killing in adult mice (Castanheira and García-Del Portillo 2017; Malik-Kale, Winfree, and Steele-Mortimer 2012). Due to physiological, structural, humoral and metabolic differences between neonate, juvenile and adult mice, the influence of these effectors in neonate NTS infections is to be determined in this study.

1.5 Aims of the study

Although *S*Ty has been studied in various *in vitro* and animal models for ages, knowledge on the mechanisms of pathogenesis, precise function of single effector proteins and interaction with host cell processes is still fragmentary. However, while a large body of *in vitro* evidence was collected over the past years, it has been difficult to pinpoint inflammatory cues and mechanisms in the infected gut *in vivo* (Hapfelmeier *et al.* 2005). Furthermore, neither *in vitro* nor animal infections fully revealed the functions of all effector proteins translocated into the host cell and workings of some virulence factors are still undefined (Figueira and Holden 2012). This study applies the neonatal mouse infection model established previously to gain insights into SPI-2 effector protein functions and their contribution to iNTS disease progression in the neonate host, especially during early steps of infection (Zhang *et al.* 2014). Unraveling mechanisms involved in the infectious process could ultimately facilitate future treatment possibilities and add to the reduction of *Salmonella* morbidity and mortality in the future.

2. Materials and Methods

2.1 Materials

2.1.1 Consumables

1.5 ml tube	SARSTEDT AG & Co. KG, Nümbrecht, Germany
15 ml tube	SARSTEDT AG & Co. KG, Nümbrecht, Germany
5 ml tube	Eppendorf, Wesseling-Berzdorf, Germany
50 ml tube	SARSTEDT AG & Co. KG, Nümbrecht, Germany
agar dish	SARSTEDT AG & Co. KG, Nümbrecht, Germany
cell strainer 100 µm	VWR International GmbH, Darmstadt, Germany
cover slide	Carl Roth, Karlsruhe, Germany
cryo viial	SARSTEDT AG & Co. KG, Nümbrecht, Germany
disposable needle	B. Braun Melsungen AG, Melsungen, Germany
disposable scalpel	B. Braun Melsungen AG, Melsungen, Germany
embedding cassette	Carl Roth, Karlsruhe, Germany
filter tip	SARSTEDT AG & Co. KG, Nümbrecht, Germany
microscope slide	Carl Roth, Karlsruhe, Germany
PCR cap stripes	SARSTEDT AG & Co. KG, Nümbrecht, Germany
PCR tube stripes	SARSTEDT AG & Co. KG, Nümbrecht, Germany
pipette tip	SARSTEDT AG & Co. KG, Nümbrecht, Germany
serological pipette	SARSTEDT AG & Co. KG, Nümbrecht, Germany
syringe filter 0.2 µm	SARSTEDT AG & Co. KG, Nümbrecht, Germany

2.1.2 Chemicals and Reagents

agarose	Bio&SELL GmbH, Nürnberg, Germany
Albumin (fraction)	Carl Roth, Karlsruhe, Germany
ampicillin	Carl Roth, Karlsruhe, Germany
chloramphenicol	Sigma Aldrich, Darmstadt, Germany
ethanol	Carl Roth, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe, Germany

glycine	Carl Roth, Karlsruhe, Germany
H2O (nuclease-free)	Carl Roth, Karlsruhe, Germany
isopropanol	Carl Roth, Karlsruhe, Germany
kanamycin	Carl Roth, Karlsruhe, Germany
LB agar	Carl Roth, Karlsruhe, Germany
Luria Broth (LB)	Carl Roth, Karlsruhe, Germany
Oligo(dT) 18 primer	eurofins Genomics, Ebersberg, Germany
paraformaldehyd/ Histofix	Carl Roth, Karlsruhe, Germany
potassium chloride	Carl Roth, Karlsruhe, Germany
potassium dihydrogenphosphate	Carl Roth, Karlsruhe, Germany
Rnase A	QIAGEN GmbH, Hilden, Germany
ROX mix	ThermoFisher Scientifics, Hennigsdorf, Germany
sodium chloride	Carl Roth, Karlsruhe, Germany
sodium citrate	Sigma Aldrich, Darmstadt, Germany
sodium hydrogen phosphate	Carl Roth, Karlsruhe, Germany
TRIzol® reagent	ThermoFisher Scientifics, Hennigsdorf, Germany
Vectashield Antifade mounting medium with DAPI	VECTOR Laboratories LTD., Peterborough, United Kingdom
xylol	Carl Roth, Karlsruhe, Germany

2.1.3 Devices

3031 shaking incubator	Edmund Bühler GmbH, Bodelshausen, Germany
3K30 cooling centrifuge	Sigma Aldrich, Darmstadt, Germany
CO_2-Incubator Heraeus Function Line	ThermoFisher Scientifics, Hennigsdorf, Germany
Fluorescence Zeiss Axiovert 200 M Inverted Microscope	Carl-Zeiss, Jena, Germany
Light microcope DMI6000 B	Leica Microsystems, Wetzlar, Germany
NanoDrop 1000	ThermoFisher Scientifics, Hennigsdorf, Germany
shaking incubator	GFL, Burgwedel, Germany
StepOne Plus Real-Time PCR system	Applied Biosystems, Hennigsdorf, Germany
sterile bench	Kendro, Langenselbold, Germany

T 10 basic ULTRA-TURRAX® homogenizer	IKA®-Werke GmbH & Co. KG, Staufen, Germany
T3000 thermocycler	Biometra, Jena, Germany
tabletop centrifuge 5415D	Eppendorf, Wesseling-Berzdorf, Germany
Ultrospec 3000 pro Photometer	Amersham Pharmacia Biotech Inc., Little Chalfont, United Kingdom
Vortex genie 2	Scientific Industries, New York, United States
water bath	GFL, Burgwedel, Germany

2.2 Methods

2.2.1 Bacterial strains and plasmids

Bacterial strains of *S. enterica* subsp. *enterica* serovar Typhimurium are listed in Table 12. Mutagenesis was performed in accordance to Datsenko and Wanner, kindly conducted by Prof. Michael Hensel, University of Osnabrück (Datsenko and Wanner 2000). The pFPV-25.1 plasmid was incorporated into competent *Salmonella* by electroporation. For selection and quantification, antibiotic resistance cassettes were applied and the respective antibiotic was added to culture media for selection as stated.

2.2.2 Cryopreservation

Liquid bacterial cultures in Luria Bertani (LB) broth containing respective antibiotics at given concentrations (Table 12) were grown at 37 °C at 200 rpm until reaching the logarithmic phase ($OD_{600\ nm}$ 0.5 – 0.6). Bacteria were harvested via centrifugation at 4000 x g for 7 min and the pellet resuspended in fresh LB medium. Bacterial solutions were mixed with sterile Glycerol to a final concentration of 20 % Glycerol in cryovials and stored at -80 °C. For re-cultivation, strains were streaked out on LB agar plates and grown overnight at 37 °C.

2.2.3 Cultivation of *Salmonella*

Bacteria were streaked out on LB agar plates containing the respective antibiotic at given concentrations and incubated at 37 °C in an incubator overnight. The next day, a single colony was picked and incubated in 5 ml LB containing antibiotics. Liquid cultures were grown at 37 °C at vigorous shaking (200 rpm) overnight and diluted 1:10 the next morning in fresh medium. Cultures were grown again until they reached logarithmic phase ($OD_{600\ nm}$ 0.5–0.6). The bacterial pellet was gained via centrifugation at 4000 x g for 7 min and washed in 1 x PBS twice. Afterwards, the pellet was thoroughly resuspended in fresh PBS, adjusted to an $OD_{600\ nm}$ 0.5 – 0.6 and diluted to the desired infectious dose. The CFU concentration was checked by serial dilution and plating on antibiotic containing LB agar plates.

2.2.4 Oral infection of neonate and juvenile mice

Adult C57BL/6N wild type and B6J.129S1-*Slc11a1*^{r}/GbrtJ (*Nramp*$^{r/r}$, stock no. 027081) mice were obtained from Charles River Laboratory (Sulzfeld, Germany) and B6.129P2(SJL)-*MyD88*$^{tm1.1Defr}$/J (*MyD88*$^{-/-}$, stock no. 009088) mice from the Jackson Laboratory (Bar Harbour, USA), respectively. Cultivation of bacterial strains was performed as described in the previous section (2.2.3). One-day old neonates were orally infected with 1 µl of the adjusted inoculum. The respective infectious doses can be abstracted from figure legends in the results section. Neonates were individualized via footpad tattoo, the weight and clinical conditions were monitored daily and assessed in accordance to an entrenched score sheet for neonate mice (Table 2).

Table 2: Scoring system for evaluating the health status of neonatal mice during infection experiments

To assess the health status and to avoid excessive stress due to the infection, a scoring system was set up. Based on distinct criteria, it allows determination of the overall health condition of an infected neonate mouse. The health status was monitored at least once daily. Mice were immediately euthanized if no body weight gain was observed over at least two days, the dam cared insufficiently (pubs placed dispersed) and motility was severely reduced to stimuli or certainly at grade 4.

grade	description	criteria				
		movement	**skin**	**fostering**	**distribution**	**weight development**
1	active	spontaneuos	pink	dam cares	pups lie together	daily weight gain (>10 %)
2	sluggish	after stimulus	pink	dam cares	pups lie together	reduced weight gain (5-10 %)
3	reduced state	reduced after stimulus	faint	dam cares	pups lie together	reduced body weight gain over two days
4	severe	motionless even after stimulus	faint and sunken	dam does not care sufficiently	dispersed in cage	no weight gain (<5 %) or weight loss

For the infection of older animals (at 6 or 11 days *post partum*, respectively) the infection volume was adjusted according to the mean weight of the litter. In order to do so, a mean weight of 1.5 g for a 1-day old mouse was estimated, corresponding to 0.66 µl bacterial solution per gram bodyweight.

At indicated time points after the infection, mice were sacrificed and organ tissue was harvested. Liver, spleen, mesenteric lymph node and colon were homogenized in sterile PBS and plated on antibiotic containing LB agar plates. Small intestines were either directly homogenized in 1 ml sterile PBS, used for primary cell isolation in case of replica plating for quantification (2.2.5) or fixed in paraformaldehyde for immunohistochemistry (2.2.7).

2.2.5 BSA/gold application to neonates to backtrace vesicular trafficking

One-day old neonates were either orally infected with a low infection dose of ΔSPI-2 *Salmonella* or left untreated. At 3 days p.i., a solution of BSA gold nanoparticles in sterile PBS was orally applied to the animals every 8 hours for 24 hours in total (corresponding to three treatments) at a volume of 8 µl per treatment. Mice were sacrificed at 4 dpi, the intestinal tracts removed, fixed in 4 % pfa for 48 hours and prepared for transelectronmicroscopic analysis kindly conducted by Urska Repnik, University of Oslo, and Dr. Mark Kühnel, Hannover Medical School as described later in this study (2.2.8).

2.2.6 Infection with tagged salmonellae

Twenty-two individual strains were tagged with a 4 nucleotide sequence, introducing an artificial stop codon into the endogenous *proV* gene as already described (Lim *et al.* 2014). Each strain was grown individually in liquid LB as stated in 2.2.3 and adjusted to an $OD_{600\,nm}$ of 0.5 corresponding to the logarithmic growth phase. Bacterial suspensions were mixed at equal ratios and sterile Glycerol (final concentration 20 %) was added to the culture suspension. The mixed inocula were shock-frozen in liquid nitrogen and stored at -80 °C until usage afterwards. Prior to infection, one aliquot was thaw on ice and used as inoculum for 5 ml LB supplemented with chloramphenicol (CM, c=10 µg/ml) overnight. The mixed tag culture was diluted 1:20 the next morning and incubated at 37 °C and 200 rpm until reaching the logarithmic phase. The culture was washed as described (2.2.3) and adjusted to an $OD_{600\,nm}$ of 0.5. To determine the total bacterial number within the inoculum, the bacterial solution was plated on CM containing LB agar plates and grown overnight at 37 °C. Colonies were counted the next morning and the inoculum on agar plates was prepared for tag sequencing as stated later in this section. The tags and their relative contribution to the reads of the inocula can be abstracted from Table 3.

Table 3: Tags applied in WITS infection of neonatal mice in and their relative contribution to the infection inocula

In total, 22 isogenic strains in the STy ATCC 14028 wt or Δ*sseB* background carried a 4 nucleotide tag in their endogenous *proV* gene to discriminate them via sequencing of samples. Unequal distribution of tags to the infection inocula was compensated by calculating an appropriate factor. By this means, the overall number of reads in the inoculum (139000 for wt and 127488 for ΔSPI-2 WITS in total) was divided by the number of tags, resulting in a theoretical tag distribution assuming equal and ideal mixtures (6318 reads per tag wt background, 5793 reads per tag ΔSPI-2 background). The actual number of reads in the inoculum was divided by this value, resulting in individual factors used for further analysis of tag distribution in infected organ tissue of neonate mice.

tag	wt background		color	ΔSPI-2 background	
	reads in inoculum	factor		reads in inoculum	factor
AAGA	5341	1.18		9555	0.61
AAGT	5458	1.16		9698	0.60
ACAA	411	15.37		312	18.57
ACCC	7552	0.84		3781	1.53
AGGC	8413	0.75		2278	2.54
ATCT	2135	2.96		5187	1.12
CAAC	10768	0.59		7367	0.79
CCAC	9304	0.68		2858	2.03
CCCC	6970	0.91		5976	0.97
CCCT	5679	1.11		11172	0.52
CGAA	20047	0.32		14678	0.39
CGGT	7993	0.79		8454	0.69
CTAC	6846	0.92		3791	1.53
GCCC	3214	1.97		2004	2.89
GGGA	2406	2.63		8178	0.71
GTGC	4487	1.41		2974	1.95
GTTA	8747	0.72		4626	1.25
TAAT	3750	1.68		6261	0.93
TAGA	8848	0.71		6948	0.83
TGCA	4753	1.33		3797	1.53
TGGA	4589	1.38		3493	1.66
TTTC	1289	4.90		4060	1.43

The WITS with the STy wt background were diluted 1:1000 in sterile PBS before oral application to 1-day old mice. SPI-2-deficient WITS were applied undiluted in a volume of 1 µl per neonate (infectious doses as stated in Figure 7). The animals were monitored daily and sacrificed at 4 days *post* infection (p.i.). Liver, spleen and mesenteric lymph node (MLN) were removed and directly homogenized in sterile PBS. The intestinal content was harvested via thoroughly rinsing the small intestinal tubes with sterile PBS and collecting the flow through. Organ material was plated on CM containing agar plates to abtain approximately 1000 CFU/plate. The plates were incubated at 37 °C overnight and formed

colonies were washed off by adding 4 ml PBS per plate and subsequently incubated at room temperature (RT) on a shaker at 300 rpm. Bacteria were collected via centrifugation and the pellet was used for gDNA isolation using Master pure kit (epicentre, Madison, Wisconsin, USA). First, the pellet was resuspended in 600 µl lysis buffer, incubated at 80 °C for 5 min and chilled on ice for 3 min. RNAse (2 µg) was added and incubated at 37 °C for one hour. Afterwards MPC Protein Precipitation Reagent (Master pure kit) was mixed with the solution and phase separation was achieved via centrifugation. Nucleic acids were washed in 70 % ethanol and the pellet containing gDNA resuspended in nuclease-free water. In the next step, the *proV* gene was amplified via PCR with primers described previously (see Appendix) (Lim *et al.* 2014). Ten ng of gDNA from each sample was used as template for a two-step PCR reaction in order to obtain an amplicon library fully compatible to the multiplexing Illumina TruSeq DNA sequencing protocol. The PCR reaction mix and protocol for initial amplification of the *proV* gene from organ homogenates are depicted in Table 4 and Table 5.

Table 4: PCR reaction mix

Ingredients for preparation of PCR in order to amplify *proV* from *Salmonella* gDNA from infection experiments of neonate mice with tagged strains (WITS).

reagent	volume	supplier
DreamTaq™ buffer (10x)	3 µl	ThermoFisher Scientifics, Hennigsdorf, Germany
proV forward primer	1 µl	ThermoFisher Scientifics, Hennigsdorf, Germany
proV reverse primer	1 µl	ThermoFisher Scientifics, Hennigsdorf, Germany
dNTPs	1 µl	Carl Roth, Karlsruhe, Germany
DreamTaq™ polymerase (5U/µl)	0.125 µl	ThermoFisher Scientifics, Hennigsdorf, Germany
gDNA	1 µl (10 ng)	-
H_2O nuclease-free	ad 30 µl	Carl Roth, Karlsruhe, Germany

Table 5: PCR protocol for amplification of *proV* from gDNA extracted from organ homogenates after mixed tag infection.

step	temperature	time	cycle repeats
initital denaturation	98 °C	2 min	
denaturation	95 °C	30 sec	40 x
annealing	60 °C	20 sec	
elongation	72 °C	30 sec	
final elongation	72 °C	2 min	
hold	4 °C	-	

The samples were sequenced on Illumina a MiSeq system using MiSeq Reagent Kits (73 cycles/323 cycles) at the Center for Biotechnology (CeBiTec) at the university of Bielefeld.

Over- or underrepresentation of individual tags due to unequal contributions to the initial inoculum were compensated by multiplying the number of reads per tag with the factor calculated beforehand (Table 3). For graphical illustration, tags with ≥1 % of reads are represented by an individual color, underrepresented tags are pooled as "others" (Figure 7).

2.2.7 Primary intestinal cell isolation

Primary intestinal epithelial cells were isolated from small intestinal tracts of orally infected neonates as previously described (Lotz *et al.* 2006). Intestinal tissue of the neonates were removed and cut into small pieces. Tissue was incubated in 30 mM EDTA PBS at 37 °C for 10 min. Vigorous shaking for 30 sec detached the epithelial cells from the underlying tissue. Cells were harvested by centrifugation at 1500 x g for 10 min at 4 °C. Afterwards, cell pellets were resuspended in 10 ml of 10% FCS/PBS and cell suspensions were passed through a 100 µm nylon cell strainer and either plated on LB containing antibiotic plates for quantification of bacteria in intestinal tissue or collected by centrifugation for transcriptional analysis afterwards (1500 x g, 10 min, 4 °C). The cell pellet was washed in PBS, collected via centrifugation, the cells were then resuspended in Trizol reagent and stored at -80 °C until usage.

2.2.8 Gene expression analysis

Total RNA was extracted from primary intestinal by phenol/chloroform extraction method. Trizol frozen cells (see previous section) were thaw on ice and incubated for 5 min. Afterwards 200 µl chloroform were added were added per tube, samples were vortexed thoroughly and then incubated at RT for 2 min, in which phase separation occurred. The samples were centrifuged at 12000 x g for 15 min at 4 °C and the RNA-containing supernatant was conferred to a new tube. Following, 500 µl Isopropanol were added and the mixture was incubated at RT for 10 min and centrifuged again at 12000 x g for 15 min at 4 °C. The resulting pellet was washed twice in ethanol and harvested by centrifugation at 7500 x g for 5 min at RT. The entire supernatant was removed and the pellet dried at RT for 15 min. Then, the RNA was dissolved in water and further employed for cDNA synthesis. In order to prepare cDNA, isolated RNA was mixed with oligo d(T) primers, incubated at 65 °C for 5 min and chilled on ice for 1 min. Afterwards, the solution was mixed with 7,5 µl Mastermix (for composition see Table 6) and incubated to cDNA synthesis at 42 °C for 1 hour, followed by a 10 min incubation period at 70 °C.

Table 6: Mastermix composition for cDNA synthesis

The ingredients for cDNA synthesis of extracted RNA and their respective volumes are listed.

reagent	volume per sample	supplier
RT buffer (5x)	4 µl	ThermoFisher Scientifics, Hennigsdorf, Germany
dNTPs	2 µl	ThermoFisher Scientifics, Hennigsdorf, Germany
Rnase inhibitor	0.5 µl	ThermoFisher Scientifics, Hennigsdorf, Germany
reverse transcriptase	1 µl	ThermoFisher Scientifics, Hennigsdorf, Germany

The cDNA was diluted with nuclease-free water and applied in gene expression analysis by Taqman based quantitative real-time PCR with the Taqman probes listed in Table 8. The gene expression was compared to uninfected, age-matched control animals and normalized to the housekeeping gene *Hprt*. Quantitative real-time PCR (qRT-PCR) was carried out with a PCR protocol described in Table 7.

Table 7: PCR protocol for Taqman based quantitative real-time PCR

Reading steps to determine threshold cycle (Ct) were set before (pre-PCR read) as well as after (post-PCR read) the whole PCR and after each annealing step.

step	temperature	time	cycle repeats
pre-PCR read	60 °C	30 sec	
initial denaturation	95 °C	15 min	
denaturation	95 °C	15 sec	40 x
annealing	60 °C	1 min	
post-PCR read	60 °C	30 sec	
hold	10 °C	-	

Table 8: Taqman probes for gene expression analysis

All probes were obtained from ThermoFisher and target murine genes. Gene expression analysis for the respective gene was always normalized to the housekeeping gene *Hprt*.

name	gene	species	assay ID	supplier
Hprt	*Hypoxanthine Phosphoribosyltransferase* 1	mouse	Mm00446968_m1	ThermoFisher Scientifics, Hennigsdorf, Germany
Cxcl5	*Chemokine (C-X-C motif) chemokine ligand 5*	mouse	Mm00436451_g1	ThermoFisher Scientifics, Hennigsdorf, Germany
Cxcl2	*Chemokine (C-X-C motif) ligand 2*	mouse	Mm00436450_m1	ThermoFisher Scientifics, Hennigsdorf, Germany
Reg3γ	*Regenerating islet-derived protein 3 gamma*	mouse	Mm00441228_m1	ThermoFisher Scientifics, Hennigsdorf, Germany
Nos2	*Nitric oxide synthase 2*	mouse	Mm00440495_g1	ThermoFisher Scientifics, Hennigsdorf, Germany
Ccl2	*Chemokine (C-C motif) ligand 2*	mouse	Mm00441242_m1	ThermoFisher Scientifics, Hennigsdorf, Germany

Saa3	*Serum amyloid A-3 precursor*	mouse	Mm00441203_m1	ThermoFisher Scientifics, Hennigsdorf, Germany

2.2.9 Immunofluorescence staining

Small intestinal tissue of neonate mice was fixed in 4 % paraformaldehyde for at least 24 hours and embedded in paraffin afterwards. Tissue was cut in 3 µm sections, deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was conducted in 10 mM sodium citrate for 10 min and blocking with 10 % serum in PBS/BSA solution prior to immunofluorescence staining. Primary antibodies (Table 9) in 5 % BSA/PBS were incubated at RT for one hour followed by washing of samples in PBS three times.

Table 9: Primary antibodies used in this study

According to the desired target structure combination to be visualized, the listed antibodies were applied in respective combinations, as stated in the figure legends within the results section.

origin species	target	dilution	supplier
rabbit	*Salmonella*	1:1000	Abcam, Cambridge, United Kingdom
mouse	E-cadherin	1:200	BD Biosciences, Heidelberg, Germany
chicken	GFP	1:500	Abcam, Cambridge, United Kingdom

Afterwards, the secondary antibodies listed in Table 10 were added at indicated concentrations in accordance to the primary antibody combination. Secondary antibodies were attached to a fluorophore and incubated on the slices for 1 hour at RT in the dark. The tissue sections were washed in PBS, counterstained with Vecta Shield mounting medium containing DAPI to stain the DNA, sealed and stored at 4 °C until usage.

Table 10: Secondary antibodies applied during this study for immunofluorescence stainings

Secondary antibodies attached to a fluorophore were chosen in accordance to the origin species of the primary antibody (Table 9).

origin species	target species	dye	dilution	supplier
goat	rabbit	Alexa Fluor 488	1:200	Jackson ImmunoResearch, West Grove, Pennsylvenia, United States
goat	mouse	Alexa Fluor 568	1:200	Jackson ImmunoResearch, West Grove, Pennsylvenia, United States
donkey	chicken	FITC	1:200	Jackson ImmunoResearch, West Grove, Pennsylvenia, United States
goat	rabbit	Alexa Fluor 568	1:1000	ThermoFisher Scientifics, Hennigsdorf, Germany

Samples were analysed in a Zeiss DMI600b fluorescence micropcope. Images were processed using LAS X software (Leica, Wetzlar, Germany) and ImageJ Fiji (NIH, USA). Pictures were adjusted for brightness and contrast.

2.2.10 Transmission electron microscopy

Infected small intestinal tissue samples obtained from neonatal mice were processed by Urska Repnik, University of Oslo. Samples were fixed in 150 mM HEPES, pH 7.35, containing 4 % formaldehyde and 0.1 % glutaraldehyde at room temperature for 1 hour and subsequently stored overnight in fixative at 4 °C. Following, samples were dehydrated in acetone and embedded in EPON. Sixty nm sections were mounted onto formvar-coated copper grids, stained with 4 % uranyl acetate and lead citrate as previously described by Reynolds and visualized in a Morgagni TEM (FEI), operated at 80 kV (Reynolds 1963). In addition, Dr. Mark Kühnel, Hannover Medical School performed transmission electron microscopy as described previously (Zhang *et al*. 2014).

2.2.11 Ethic statement

All animals were handled in accordance with regulations defined by FELASA and the national animal welfare body GV-SOLAS. *In vivo* experiments were performed in compliance with the German animal protection law (TierSchG) and approved by the local animal welfare committee. Approvals G0293/15 and T0284/15 of the Landesamt für Gesundheit und Soziales Berlin, Germany, 33.14.42502-04-12/0693 of the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit Oldenburg, Germany, as well as 81-02.04.2017.A397 of the Landesamt für Natur, Umwelt und Verbraucherschutz, North Rhine Westfalia.

2.2.12 Statistical analysis

Any statistical analysis of data during this study was conducted using GraphPad Prism 8 software. Results of bacterial growth in organ tissues and gene expression analysis display individual animals plus the median if not indicated otherwise For comparison of two independent groups, student's t-test with Mann-Whitney-U-test was applied, whereas statistical differences of data sets within experiments containing three or more distinct groups were determined via one-way ANOVA and Dunn's post-test. In either case, differences $p \geq 0.05$ were considered not significant (ns), * $p \geq 0.01$, ** $p \geq 0.001$, *** $p \geq 0.0001$, **** $p \leq 0.0001$, as indicated in the corresponding figure legends.

3. Results

3.1 Functions of SPI-2 in the neonate host

The effector proteins encoded by the *Salmonella* pathogenicity island-2 (SPI-2) are known to be a requirement for development of systemic virulence in adult mice for ages (Coburn *et al.* 2005). We were particularly interested in the properties of SPI-2 effectors in the pathogenesis of NTS infections in the neonate host and therefore compared virulence of the SPI-2-deficient STy mutant versus the wildtype strain in the first place.

3.1.1 SPI-2 effector protein translocation is crucial for systemic spread in neonates

After oral application to neonate mice, the ΔSPI-2 mutant *S.* Typhimurium (STy) strain showed significantly attenuated virulence in infected mice within the first 11 days after infection, whereas more than 50 % (n=10) of animals infected with the same dose of wt STy succumbed to the causes of the infection within the first 5 days (Figure 3 A). Correspondingly, bacterial loads of systemic tissue as well as the mesenteric lymph node (MLN) were markedly reduced in the mutant as compared to the wt strain ($p>0.0001$, $p=0.0023$, $p>0.0005$, Figure 3 B-D). In stark contrast to this observation, colonization of the small intestinal enterocytes (IECs) and colon are highly similar between wt and SPI-2-deficient bacteria (Figure 3 E-F). By trans-complementation, the virulent systemic phenotype of the wt strain with increased bacterial loads of liver and spleen was restored again.

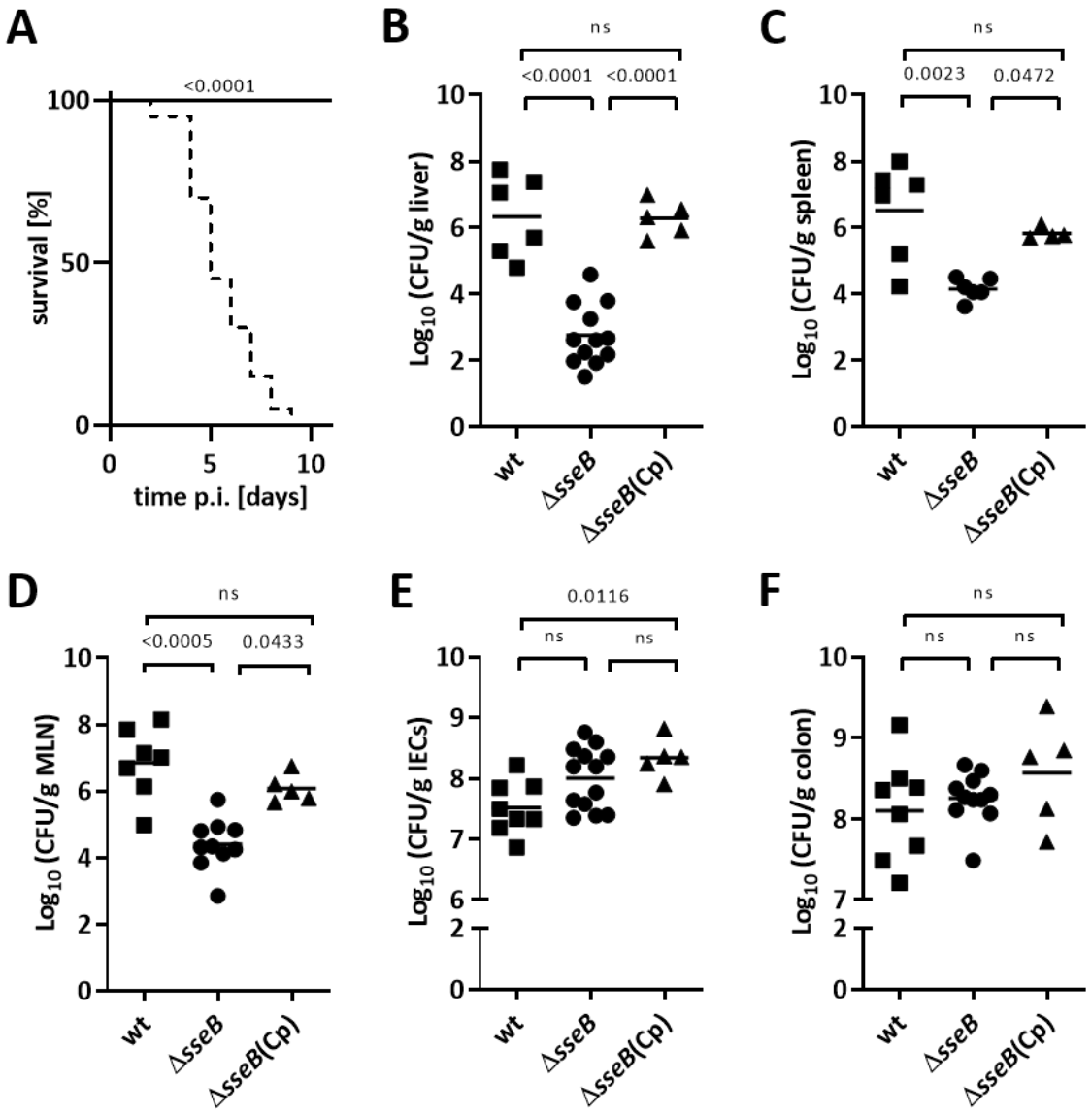

Figure 3: SPI-2 is essential for development of systemic virulence in the neonate host.

(A) Neonate mice orally infected with 10^2 CFU wt (dashed line, n=20) or SPI-2-deficient *Salmonella* (solid line, n=18) were monitored daily for their degree of severity according to a well-established scoring system (Table 2) and the survival rate was calculated in accordance. Four days p.i. (dpi), mice infected at 1 day *post partum* (*p.p.*) with wt (squares), Δ*sseB* STy (circles) or a complemented Δ*sseB* mutant strain (triangles) were sacrificed and replica plating of liver (B), spleen (C), mesenteric lymph node (MLN, D), intestinal enterocytes (IECs, E) and colon (F) homogenates revealed bacterial loads of different tissue compartments. ANOVA with Dunn's multiple post-test was applied to calculate statistical significances as indicated, a p-value >0.05 is indicated by "ns".

3.1.2 The influence of host maturation on SPI-2-independent infection

In the adult murine model, attenuated virulence of ΔSPI-2 STy after oral inoculation has been accompanied by the inability to disseminate to systemic sites (Shea *et al.* 1999). In these animals, colonization resistance is overcome by antibiotic treatment prior to infection (Kaiser *et al.* 2012), a redundant step in neonatal mice that have not established a protective intestinal microbiota thus far. Infection of slightly older, but still suckling mice with a weight adapted inoculum of ΔSPI-2 STy did not

yield detectable bacterial load in neither liver nor spleen (p=0.0004, p>0.0001, Figure 4 A-B). In addition, the number of mutant bacteria in the MLN as well as SI and isolated enterocytes was significantly reduced when compared to the neonatal infection (Figure 4 C-E). Different anatomical distributions of ΔSPI-2 bacteria became more apparent with increasing host age. Whereas both the proximal and distal part of the intestine are similarly loaded after a neonatal Δ*sseB* STy infection, the distal part displayed higher bacterial densities than the proximal part in both intestinal enterocytes and whole organ homogenates of older mice infected at 6 and 11 days *post partum* (*p.p.*) (Figure 4 F-G). Overall, bacterial burden of the intestinal enterocytes was markedly reduced in older mice, as compared to neonates (p=0.0196, p>0.0001).

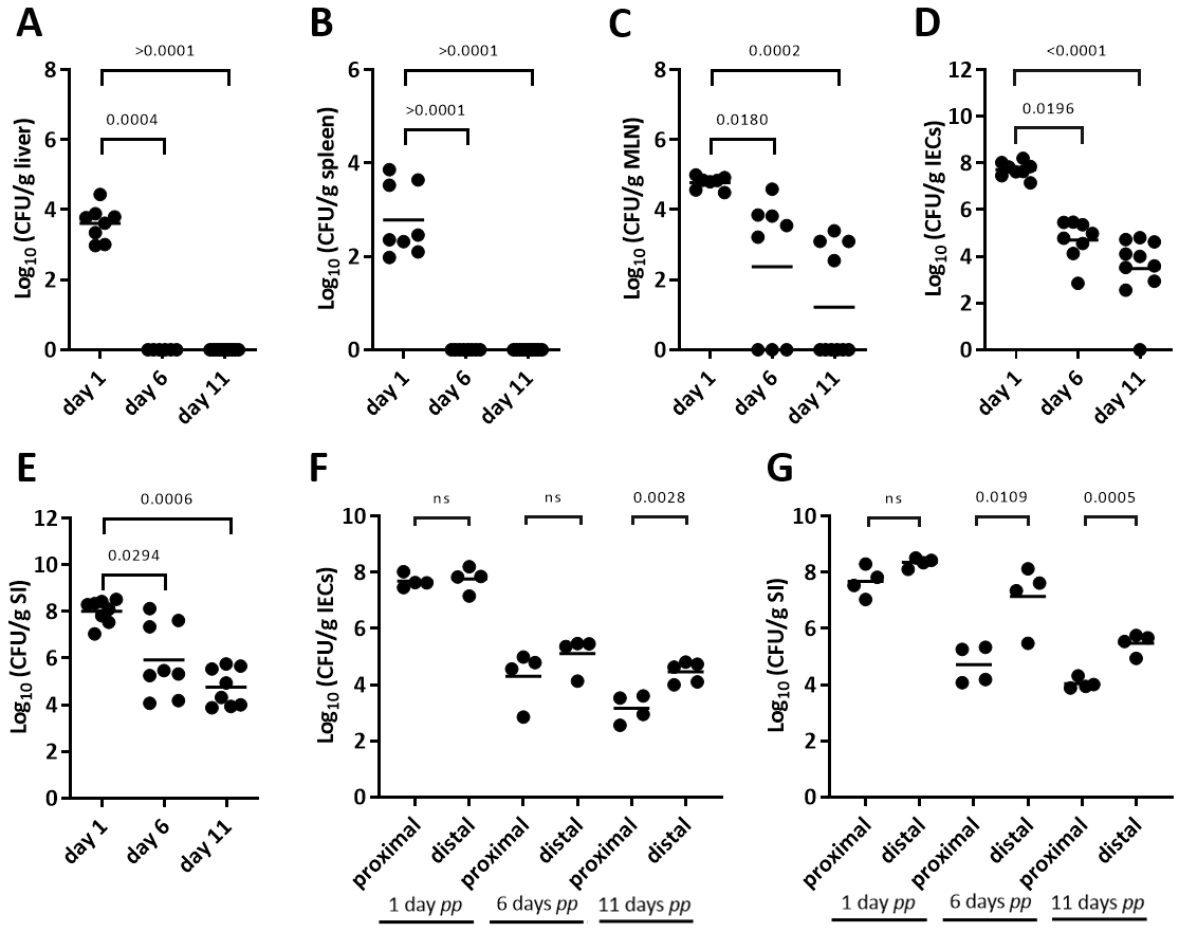

Figure 4: Host maturation influences pathogenesis in the absence of SPI-2.

Mice were orally infected with a weight-adapted dose of Δ*sseB Salmonella* at 1, 6 or 11 days *p.p.*. At 4 days p.i., mice were sacrificed and organ loads were determined by replica plating for liver (A), spleen (B) and MLN (C). Concerning the small intestine (SI), whole organ homogenates (E+G) were compared to isolated intestinal enterocytes (D+F) from the bisected other half of the SI of the very same animal. ANOVA with Dunn´s multiple post-test was used for statistical analysis, a p-value ≥ 0.05 is considered not significant, indicated as "ns". The bacterial load of the proximal part of the SI and IECs was compared to the distal part of the same compartment (F-G). Student`s t-test was used for statistics.

3.1.3 *Salmonella*-induced inflammation in the neonate gut

After depletion of the resident microbiota by streptomycin treatment (Barthel *et al.* 2003), the invasion of primarily M (microfold) cells in the gut (Haraga, Ohlson, and Miller 2008) by *Salmonella* induces massive inflammation and upregulation of different cytokines in adult mice (Kaiser *et al.* 2012; LaRock, Chaudhary, and Miller 2015; Miki *et al.* 2017; Stecher *et al.* 2005). This ultimately leads to the recruitment of immune cells to the *lamina propria* underlying the epithelial barrier in the intestinal tract, probably accelerating systemic dissemination of the pathogen (Cheminay, Chakravortty, and Hensel 2004). Hence, we next wondered whether an inability of ΔSPI-2 mutant bacteria to cause inflammation might explain diminished systemic spread and attenuated virulence in the neonate host at least to some degree (3.1.1).

Similar to progression of NTS infections in adult mice, infection of neonate mice with wt *Salmonella* yielded a significant upregulation of *Cxcl5* (p=0.033), *Cxcl2* (p=0.0004), *RegIIIγ* (p=0.0053), *Nos2* (p=0.0017) and *Saa3* (p=0.0016), but not *Ccl2* (Figure 5 A-F). In contrast, in the absence of SPI-2, only certain antimicrobial factors were significantly higher expressed compared to the uninfected control (*RegIIIγ,* p=0.0417; *Nos2,* p=0.0225 and *Saa3*, p=0.0193), whereas other cytokines tested were expressed to a similar extent (Figure 5 A-F). Although the SPI-2-deficient mutant did not cause any mortality (Figure 3 A), a dramatic induction of *RegIIIγ* expression was caused by infection of the neonate host. Peaking at 4 dpi, *RegIIIγ* levels remained elevated, but simultaneously, expression also increased under physiological conditions with maturation of the host (Figure 5 G). These processes lead to similar R*egIIIγ* levels in ΔSPI-2-infected and uninfected mice at the age of 19 days *p.p.* (Figure 5 G).

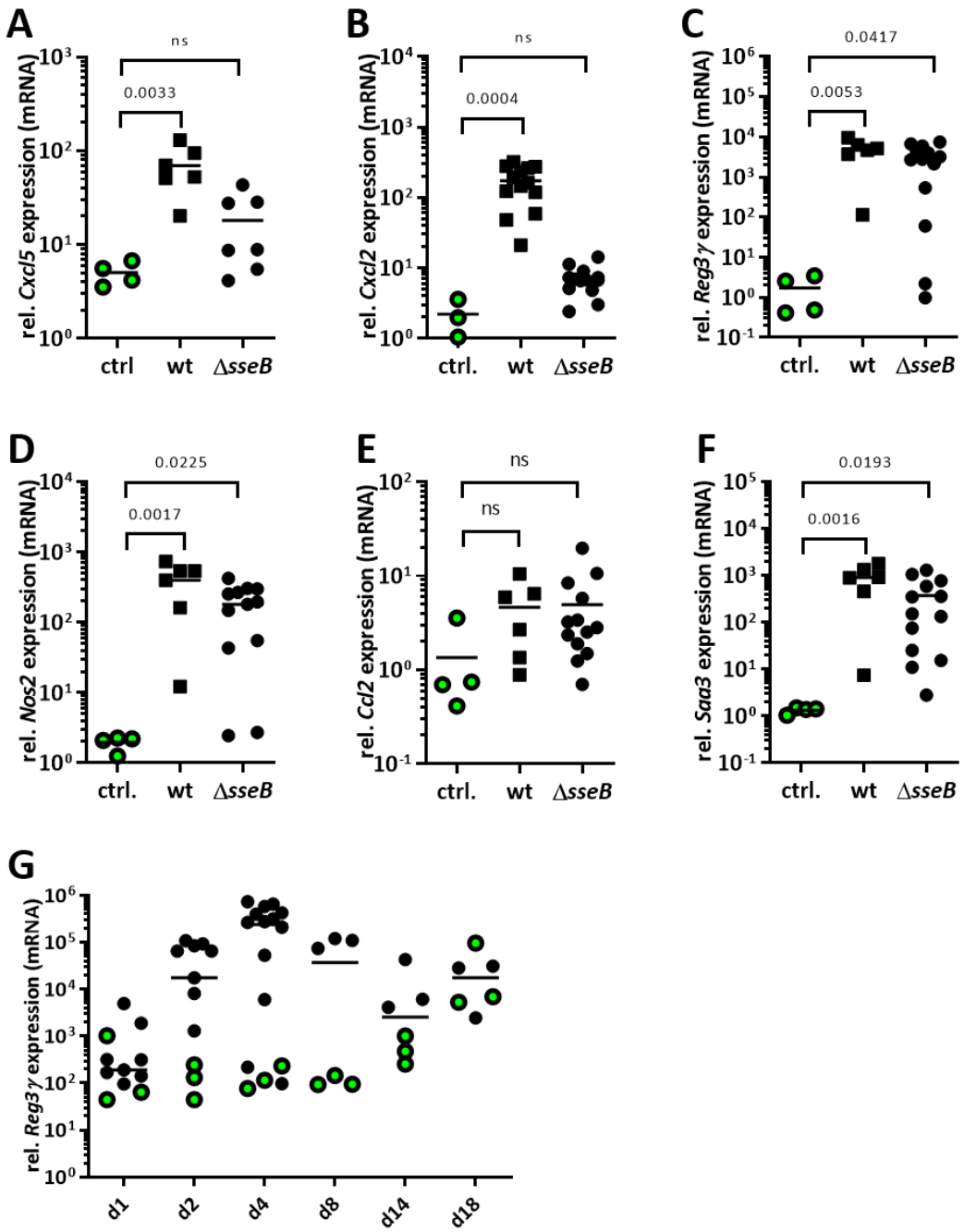

Figure 5: SPI-2-deficient *Salmonella* induce intestinal inflammation in the neonate gut.

Quantitative RT PCR of uninfected control mice (ctrl, green circles) and mice neonatally infected with either 10^2 CFU wt (squares) or SPI-2-deficient *Salmonella* (circles) at 4 dpi. Expression of C-X-C motif chemokine 5 (*Cxcl5*, A), *Cxcl2* (B), regenerating islet-derived protein 3 gamma (*RegIIIγ*, C), inducible nitric oxide synthase 2 (*Nos2*, D), CC-chemokin-ligand-2 (*Ccl2*, E) and serum amyloid A 3 (*Saa3*, F) normalized to the expression of the housekeeping gene *Hprt* is depicted as relative (rel.) expression. ANOVA with Dunn´s multiple post test was used for statistical analysis, a p-value ≥ 0.05 is considered not significant and indicated as "ns". Additionaly, quantitative RT PCR for expression of *RegIIIγ* mRNA in intestinal enterocytes isolated at indicated time points after neonatal infection with ΔSPI-2 STy was performed (G). One-day old mice were infected with 10^2 CFU ΔSPI-2 mutants (black circles) or age-matched uninfected control animals, which were left untreated (green circles). The given results represent the median values from two independent experiments (n=8 per group infected, n=3 uninfected control animals). The expression was normalized to the housekeeping gene *Hprt*.

3.2 The peculiar phenotype of SPI-2-deficient *Salmonella*

The colonization rate of the small intestine and colon of neonatal mice infected with wt or SPI-2-deficient *Salmonella* was highly comparable (Figure 3 D-E). Earlier studies demonstrated expression and functional secretion of SPI-2 to be a necessity for intracellular survival and replication of STy in isolated macrophages (Cirillo *et al.* 1998) and epithelial cell lines (Groisman and Ochman 1996). We next aimed at the further clarification of the consequences of lacking SPI-2 effectors in the neonate intestine in more detail.

3.2.1 SPI-2-independent SCV formation in the neonatal small intestine

To minimize histological changes caused by the progressive infection and inflammation in wt infected neonates (Figure 3 B-D, Figure 5 A-F), we focused on a time point at 4 dpi. for further investigations, where the majority of wt infected mice displayed clear signs of systemic infection, but did not succumb to the consequences yet (Figure 3 A). Immunofluorescence staining of tissue sections from wt and ΔsseB infected neonates at this time point clearly indicates the accumulation of mutant bacteria inside an infected epithelial cell, as holds true for wt *Salmonella* (Figure 6 A). After invasion of a non-phagocytic intestinal epithelial cell by translocation of SPI-1 effectors (Zhang *et al.* 2014), *Salmonella* replicates within a specialized, membrane-bound compartment known as the *Salmonella*-containing vacuole (SCV, 1.4.2) (Vazquez-Torres, Xu, *et al.* 2000). Although *sseB*-deficient *Salmonella* are unable to translocate the entire SPI-2 effector protein subset, SCV formation does not seem to be impaired in the neonate gut (Figure 6 B). Furthermore, SCVs formed by mutant bacteria are approximately 5 times bigger than vacuoles of wt bacteria at the same time point ($p>0.0001$, Figure 6 C). Transmission electron microscopic pictures of ΔSPI-2-infected cells in the neonate small intestine encourage the finding of an intact vacuole surrounding various mutants inside an infected enterocyte (Figure 6 D). Apposite to the attenuated virulence of mutant as compared to the wt strain, the brush border of infected cells remained completely intact (Figure 6 D iii). Although functional translocation of effector proteins from the inside of the SCVs into the host cell cytosol is no longer possible in the mutant due to a fragmentary translocon (Figure 2), expression of SPI-2 is not impaired and intracellular expression of effector proteins remains functional, demonstrated by infection with a GFP-reporter strain (Figure 6 E). This proves intact SCV formation in the small intestine of neonatal mice in the absence of SPI-2 effector protein translocation.

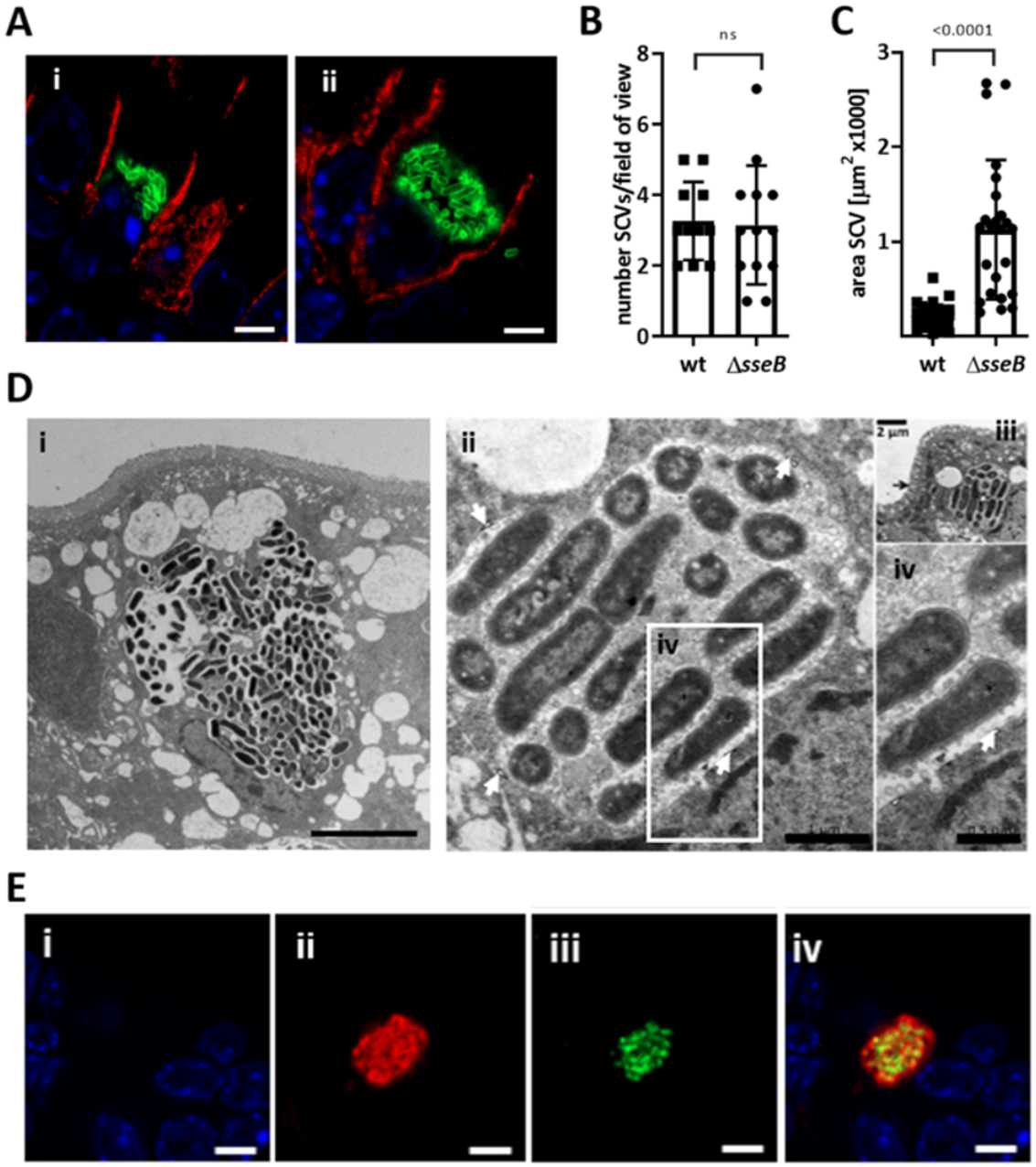

Figure 6: SCV formation in the neonate gut is SPI-2-independent.

Immunofluorescence of small intestinal tissue sections of wt (i) and ΔSPI-2 STy (ii) infected mice 4 days after oral administration reveals bacterial accumulation, that can be considered as Salmonella-containing vacuoles (SCVs) inside infected enterocytes in both types of infection (A). Scale bars represent 5 µm. The compare of the number (B) and size (C) of SCVs in small intestinal sections of wt and ΔSPI-2 STy reveals no significant difference in SCV formation between wt and mutant bacteria. At least 20 fields of view from 3 different animals were investigated for determination of SCV number and at least 40 SCVs from 3 different animals were measured for SCV sizes. Results represent median values, significance was calculated with the student's t-test ns, $p>0.05$. Transmission electron microscopy pictures of SCVs from *Salmonella* ΔSPI-2 infected mice by Dr. Mark Kühnel also hint to membrane enclosed accumulations of bacteria inside an infected host cell (D). White arrows in (ii) and (iv) indicate an intact membrane entirely surrounding the SCV. Bars represent 20 µm (i), 1 µm (ii), 2 µm (iii) and 0.5 µm (iv). Immunostainings of SPI-2-deficient *Salmonella* carrying a *gfp*-reporter allele under the control of the promoter of the SPI-2 effector protein SsaG. Single channels of DNA (i, blue),

Salmonella (ii, red) GFP (iii, green) and a merged channel image (iv) are depicted (E). Bars represent 10 µm.

3.2.2 The absence of SPI-2 influences dissemination and distribution of subpopulations in invasive NTS infections

Expansion of knowledge regarding pathogenesis of invasive *Salmonella* infections has progressively gathered research attention. A study by Lim and colleagues made use of mixed infection with tagged *Salmonella* to reveal dynamics of the infection process with special respect to bottleneck formation in distinct steps of pathogenesis and distribution of tagged *Salmonella* in different tissue compartments (Lim *et al.* 2014). We utilized mixed infection with individually tagged wt and ΔSPI-2 *Salmonella* in order to prove improper transmigration of the epithelial barrier in the neonate small intestine by the mutant, resulting in diminished systemic spread and attenuated virulence.

Dissemination of wt and SPI-2-deficient tagged *Salmonella* was investigated in neonatal mice at 4 days p.i. in the next step. As not all 22 subpopulations contributed equally to the infection inoculum, their relative proportion was calculated and considered for further evaluation. In order to do so, the number of reads of an individual tag was multiplied with a factor calculated beforehand to compensate for over- or underrepresentation already present in the inoculum (see Table 3, Figure 7 A). Distributions of individual tagged bacteria in different organ compartments of infected mice are depicted in pie diagrams (Figure 7 B-C). Only those tags with more than 1 % of the overall reads per sample were taken into account and are displayed with a distinct color in the pie diagram, whereas underrepresented samples are summarized in grey.

Infection of neonate mice by *Salmonella* and especially the colonization of the intestine as the first step in pathogenesis obviously represents a process of non-Darwinian selection, since the isolated tags between different animals in the same compartment can vary strikingly (e.g. wt infected animal IV vs V). Besides this, overrepresentation of a subpopulation of WITS in the intestinal tract does not necessarily lead to an equal proportion of it in systemic organs for neither wt nor SPI-2-deficient mutant infection.

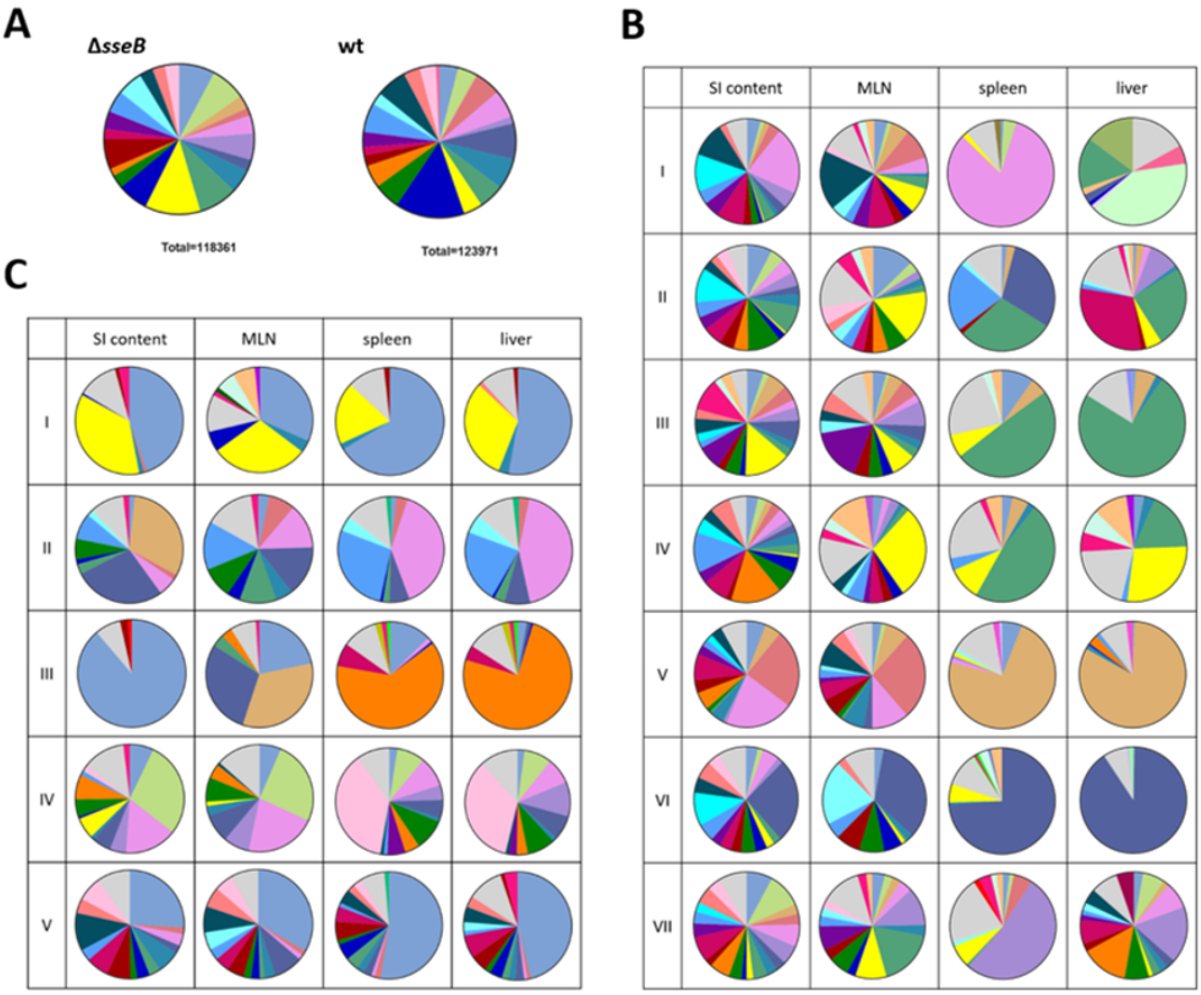

Figure 7: Population dynamics in neonatal invasive NTS infection

Neonatal mice were orally infected with 10^4 CFU Δ*sseB* or 10^3 CFU wt *Salmonella*. The different contribution of each of the 22 individually tagged subpopulations in the inoculum is depicted in A. Each colour represents a distinct tag (Table 3). At 4 days p.i., homogenates of liver, spleen and MLN as well as small intestinal flushings were plated and all colonies harvested by washing. gDNA was isolated and the *proV* gene amplified. The amplicons were sequenced by MiSeq. Pie diagrams depict the relative proportion (2.2.4.2, Table 3) of the different SPI-2-deficient (B, n=7) or wt (C, n=5) WITS in investigated organ compartments of individual animals labeled I-VII or I-V, respectively, with all compartments of one animal in the same row. Each color represents an individual tag conducing to more than 1 % to the overall reads per sample. Underrepresented tags are collectively shown in grey as "others".

The different *Salmonella* subpopulations within distinct tissue compartments of the same animal were compared in more detail afterwards to investigate the role of SPI-2 in pathogenesis and systemic spread a little further. Hence, the individual tags detected in more than one organ within the same host and the resulting sequence identity among distinct tissues is contrasted between wt and Δ*sseB* infection (Figure 8 A-D). For the wt infection, sequence identities are high, especially between liver and spleen (mean value 88 % tag identity, Figure 8 A) and moderate for liver vs SI content (mean value 64 % tag identity, Figure 8 B), spleen vs SI content (mean value 69 % tag identity, Figure 8 C) and MLN vs SI content (mean value 66 % tag identity, Figure 8 D). In contrast to this, identities between different

organs within the same host infected with SPI-2-deficient strains are markedly decreased (mean values tag identity: liver vs spleen 35 %, liver vs SI content 33 %, spleen vs SI content 25 % and MLN vs SI content 60 %). As already indicated in Figure 7, populations and distribution of different tags among distinct tissues clearly show overlaps and similarities for wt-infected neonates, whereas subpopulations in organs of Δ*sseB*-infected mice are highly different, especially when comparing SI content to systemic compartments. By comparison of tag identities, this observation could be further validated.

The colonization of systemic organs, lymphoid tissue and small intestinal enterocytes during early phases of the infection with tagged *Salmonella* is shown in Figure 8 E-H. At 2 dpi, bacterial burden in liver (Figure 8 E), spleen (Figure 8 F) and IECs (Figure 8 H) is similar in wt and SPI-2-deficient WITS and slightly increased in the mutant infection in the MLN (Figure 8 G). Between 2 and 4 dpi, a significant increase in the number of wt bacteria could be detected in all investigated organ compartments that was absent in the SPI-2-depleted tagged strains. This suggests an initial dissemination event after oral infection independent of SPI-2 effector protein translocation that is fueled during disease progression via SPI-2 effectors. Bearing in mind previous experiments, this seems to be limited to the neonatal period (Figure 4). Considering the different distribution of subpopulations between the two types of infection (Figure 7), a bottleneck for invading *Salmonella* seems to be imposed in neonates beyond the gut, similar to pathogenesis in adult mice (Lim *et al.* 2014). Overall, populations among different organs display high similarities in wt-background WITS infection, whereas subpopulations in systemic organs are distinct from the tag distribution in the intestine and MLN in the absence of SPI-2.

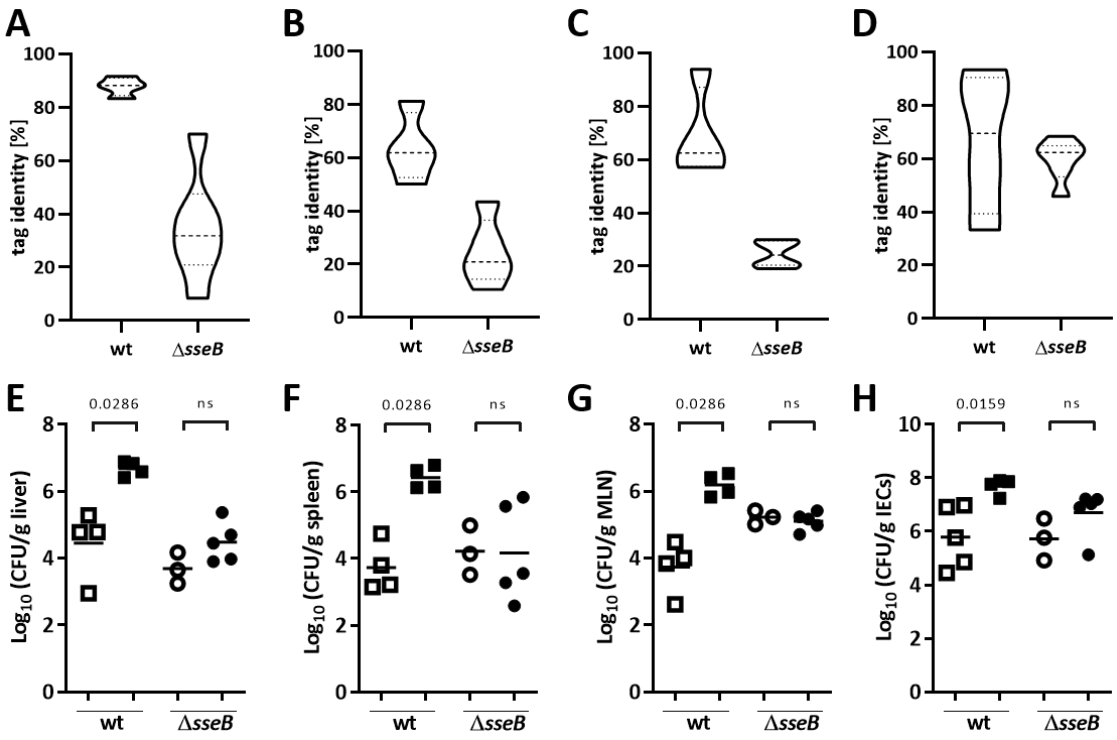

Figure 8: SPI-2 effectors strongly influence systemic distribution of *Salmonella*.

One-day old mice were orally infected with 10^4 (A-D) or 10^3 (E-H) CFU Δ*sseB* or 10^3 CFU wt *Salmonella*. The identity of tags from mixed infection with indivudually tagged *Salmonella* in the wt or ΔSPI-2 background among different organs of the same animal are shown to display differences or simiartities in the distributions of subpopulations in the different tissues by violin diagrams. Only those tags with more than 1% of the reads were considered and their degree of identity between liver and spleen (A), liver and SI content (B), spleen and SI content (C) or MLN and SI content (D) was calculated per infection. Given plots respresent the median values (bold dashed line) and the sample distribution is indicated by the different quantiles (dotted lines within violins). The number of bacteria in different organs at 2 (open symbols) and 4 dpi (closed symbols) after oral infection with tagged strains are compared between wt (squares) and Δ*sseB* mutant (circles) in liver (E), spleen (F), MLN (G) and SI content (H). Student`s t-test to determine significant differences, a p-value ≥0.05 is displayed by labelling "ns".

3.2.3 MYD88 is not required for intracellular replication of ΔSPI-2 bacteria

Neonates, in contrast to adults, harbor an immature immune system, especially regarding the adaptive immune response. As the gut is probably the most important interface between the sterile body interior and the environment, fast reactivity to invading pathogens is of particular importance for host survival. Especially during early stages of an infection, the innate immune system is highly essential in controlling replication of invading bacteria and their spread before an adaptive response can be initiated. Many TLRs, as potent receptors of the innate immune response, are known to be involved in recognition of *Salmonella*, including TLR2 (Tukel *et al.* 2005), TLR4 (O'Brien *et al.* 1980) and TLR5 (Feuillet *et al.* 2006; Fulde *et al.* 2018; Uematsu *et al.* 2006). Hence, knockout of single TLRs or their

adapter protein myeloid differentiation primary response 88 (MYD88) ultimately yields increased susceptibility in orally exposed adult mice (Sivick *et al.* 2014). In addition, the expression of SPI-2 and intracellular replication as well as SCV formation has been stated to be dependent on the acidification of the SCV, conceivably achieved via MYD88-dependent signaling cascades in bone marrow macrophages (BMMs) (Arpaia *et al.* 2011; Hapfelmeier *et al.* 2005). Due to these previous observations, we aimed at the identification of the role of MYD88-dependent TLR-signaling in neonate mice after SPI-2 mutant infection in the next step.

The oral infection of *Myd88* knockout (KO) mice with ΔSPI-2 *Salmonella* yielded comparable bacterial burden in the systemic organs as compared to B6N wildtype mice infected with the mutant strain. Slightly higher bacterial burden was detected in the spleens of *Myd88* KO neonates ($p=0.0043$, Figure 9 B), whereas colonization of liver, MLN and colon are unaffected in the absence of MYD88-signaling cascades. More mutants could be isolated from small intestinal tissue of *Myd88*-deficient neonates ($p=0.0289$, Figure 9 D). These findings already indicate that possession and secretion of SPI-2 effectors is non-essential for bacterial survival even in the absence of MYD88. We next examined whether a lack of downstream TLR-signaling via MYD88 influences the capability to establish and maintain SCVs inside infected host cells in the neonatal intestine, as has been suggested in earlier studies for BMMs (Arpaia *et al.* 2011). Immunofluorescence of ΔSPI-2 infected small intestinal sections of neonatally infected *Myd88*$^{-/-}$ mice validates SCV formation without MYD88-dependent TLR-signaling at 4 days p.i. (Figure 9 F-H). Neither the number of SCVs (Figure 9 F) nor their size (Figure 9 G) differed significantly from SCV formation in B6N neonates with intact MYD88 cascade, identifying the adaptor protein as non-essential in terms of SCV development in the neonate gut.

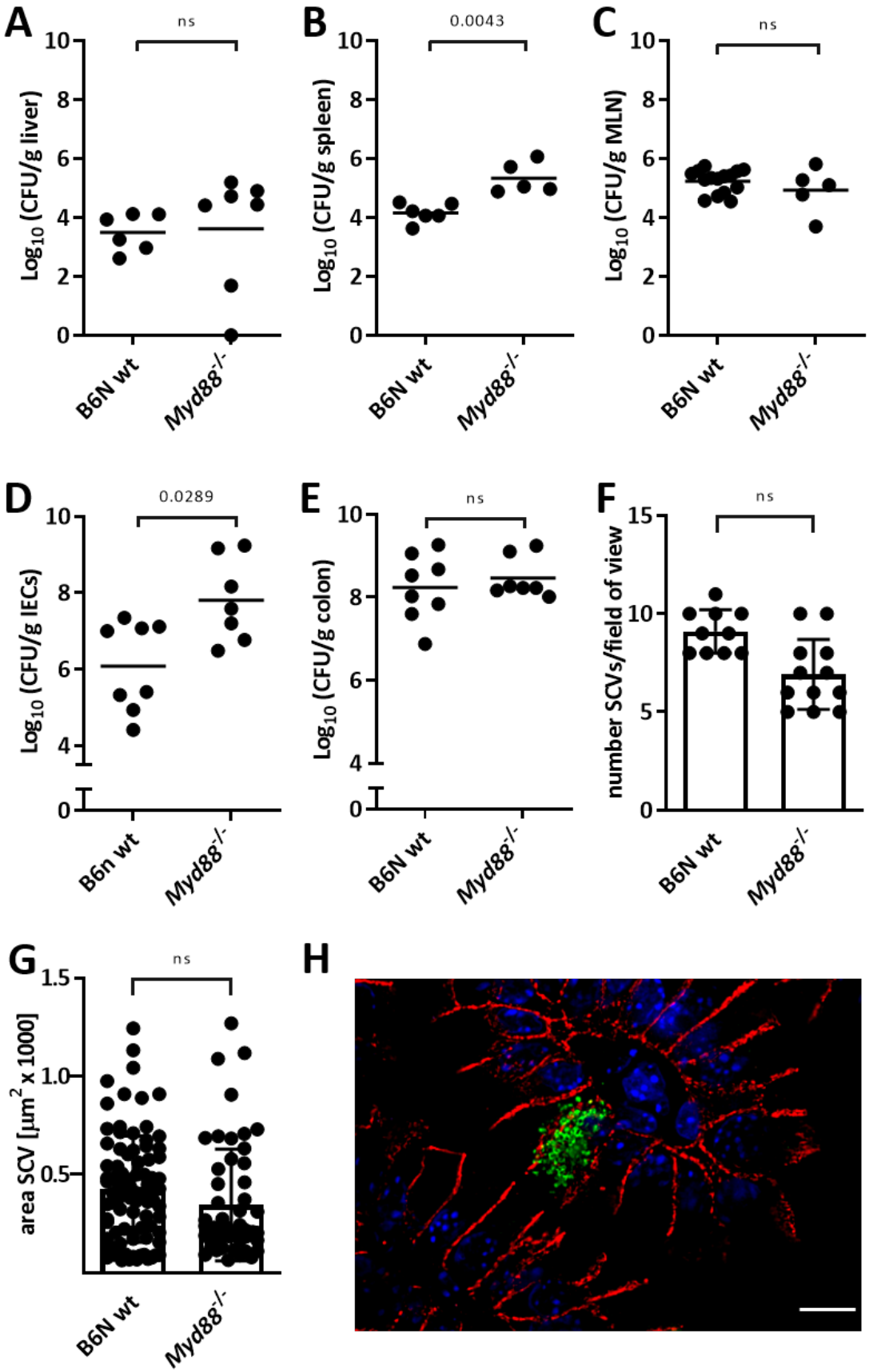

Figure 9: ΔSPI-2 STy can colonize the neonate gut and form SCVs in the absence of MYD88.

One-day old B6N wt or knockout mice lacking the adaptor protein MYD88 were orally infected with 10^2 CFU Δ*sseB* STy. Four days p.i., mice were euthanized, and bacterial loads investigated via replica

plating in liver (A), spleen (B), MLN (C), isolated intestinal enterocytes (D) and colon (E). Small intestinal tissue sections were stained as described (2.2.7) and the number (F) of SCVs in at least 20 fields of view from 3 different animals were determined, plus at leat 40 SCVs from 3 different animals for SCV size calculation (G). Student's t-test with Mann-Whitney-U was used for statistical analysis to compare KO to B6N wt mice infected with ΔSPI-2 mutants, p≥0.05 is indicated by "ns". Immunfluorescence of SCVs formed by ΔSPI-2 bacteria in *Myd88*$^{-/-}$ mice (H) reveals no difference in SCV formation capabilities of the mutant in the absence of MYD88 compared to B6N wt mice (Figure 6 A ii). *Salmonella* shown in green, E-cadherin in red, counterstaining with DAPI for DNA in blue, scalebar corresponds to 10 µm.

3.2.4 The influence of functional NRAMP1 on NTS dissemination beyond the gut

The commonly used mouse strain C57BL/6 allows versatile manipulations of host genetics in terms of receptors like TLRs, cytokines like e.g. interleukins or other messengers and mediators of the adaptive and innate immune response. However, one major shortcoming in the examination of pathogenesis of intracellular bacteria is a point mutation in *Slc11a1* gene, leading to nonfunctional expression of natural resistance-associated macrophage protein 1 (NRAMP1), expressed primarily in late phagolysosomes of macrophages, but also in neutrophils and DCs (Blackwell *et al.* 2000; Canonne-Hergaux *et al.* 2002; Forbes and Gros 2001). Mutations, e.g. by an amino acid substitution in C57BL/6 and BALB/c mice resulting in a non-functional protein, have been associated with the innate susceptibility of mice to infection with a group of unrelated intracellular parasites, including *Mycobacterium bovis*, *Salmonella* Typhimurium, and *Leishmania donovani* (Vidal *et al.* 1995). Furthermore, polymorphisms in *Nramp1* were demonstrated in association with increased susceptibility to tuberculosis and certain autoimmune disorders in humans (Bellamy *et al.* 1998; Blackwell *et al.* 2001). Consequently, we were interested in the degree of influence of a functional NRAMP1 in the neonate host and especially in NRAMP1-mediated killing influenced by the presence or absence of SPI-2.

As expected, comparison of wt *Salmonella* infection in B6N wt (NRAMP1 negative) and *Nramp*$^{+}$ age-matched mice revealed significantly lower viable bacterial counts in systemic tissue in the presence of functional NRAMP1 ($p<0.0001$ and $p=0.0004$, Figure 10 A-B), whereas colonization of the gut and mesenteric lymph node is unaffected by the presence or absence of a functional allele (Figure 10 C-E). In contrast to this, if SPI-2 is depleted, functional NRAMP1 is irrelevant for bacterial burden in any investigated tissue (Figure 10 A-E). Overall, this indicates that the pathogen's virulence factor set rather than host genetic background determines the outcome of NTS infection of the neonate host.

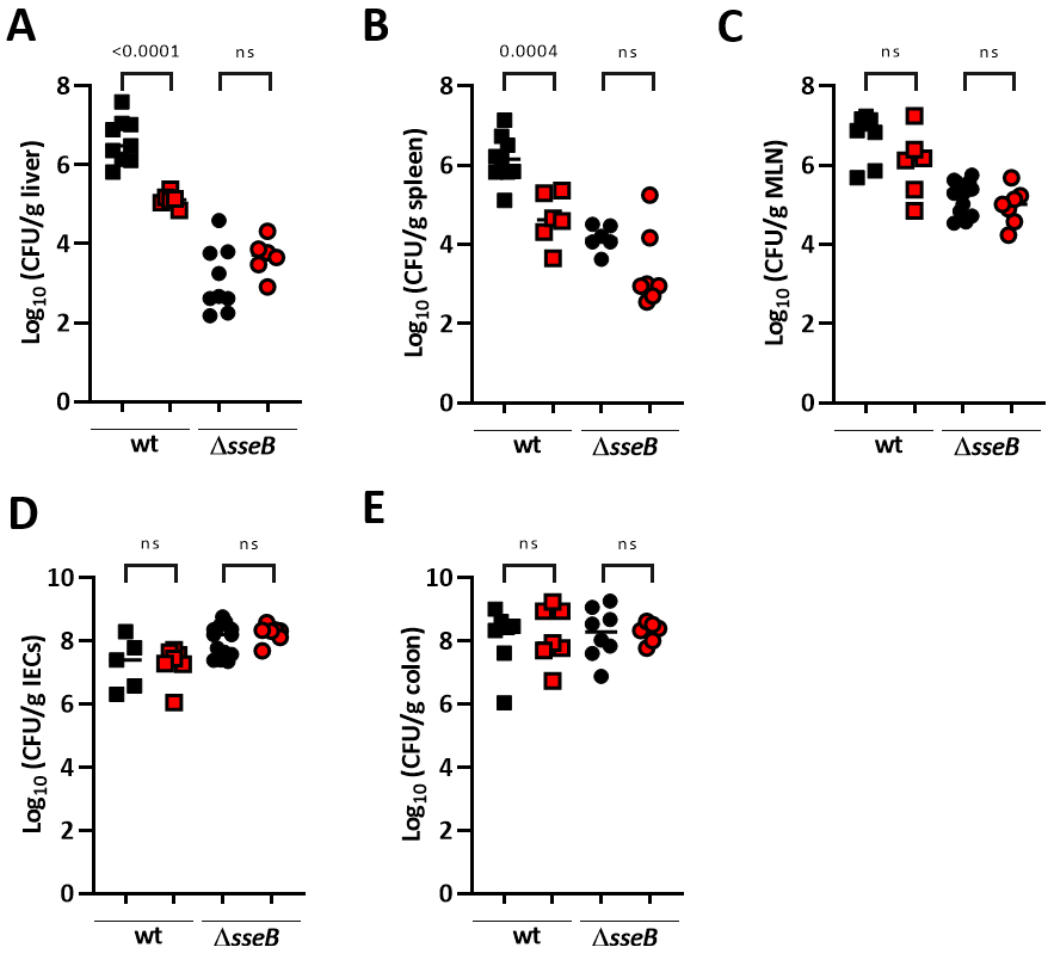

Figure 10: Functions of NRAMP1 in wt and Δ*sseB* STy pathogenesis

One-day old C57BL/6N (black symbols) or *Nramp1*$^{r/r}$ mice harboring a functional *Nramp1* allele (red symbols) were orally challenged with 10^2 CFU wt (squares) or SPI-2-deficient (circles) *Salmonella*. At 4 days p.i., mice were sacrificed and the bacterial burden of given organ compartments was determined via replica plating for liver (A), spleen (B), MLN (C), IECs (D) and colon (E).

Table 11: Ratio of wt vs ΔSPI-2 *Salmonella* in B6N and NRAMP1$^+$ neonatal mice

Results from oral infection experiments with wt and ΔSPI-2 *Salmonella* in B6N and *Nramp1*$^+$ mice as ratios between wt STy CFU/ml compared to Δ*sseB* CFU/ml (Figure 10). A ratio of e.g. 2.28 indicates 2.28 x more wt bacteria in the respective organ as compared to tissue isolated from neonates infected with the mutant strain. The mean of each column is shown in bold letters in the last row.

liver		spleen		MLN		SI		colon	
B6N	*Nramp*$^+$	B6N	*Nramp*$^+$	B6N	*Nramp*$^+$	B6N	*Nramp*$^+$	B6N	*Nramp*$^+$
5.68	11.34	2.28	0.57	4.84	1.90	0.11	0.33	5.55	4.49
1.09	18.43	545.63	0.11	17.78	17.36	0.15	0.44	0.28	0.30
409.39	17.64	212.80	2.53	512.88	17.43	0.28	0.44	1.35	2.60
1069.99	16.69	436.51	0.64	66.51	1.45	0.96	0.17	1.05	0.35
214.00	7.95	76.39	1.07	24.73	1.48	0.49	0.01	0.06	0.02
9.89	15.59	4.91	2.16	84.48	17.12	0.14	0.30	0.50	0.09
225.01	14.61	213.09	1.09	101.62	9.41	0.04	0.19	2.38	8.26
276.44	**12.82**	**209.77**	**1.18**	**116.12**	**9.46**	**0.31**	**0.27**	**1.60**	**2.30**

3.3 SCV formation and maintenance in the absence of SPI-2

3.3.1 ΔSPI-2 *Salmonella* stably maintain SCVs during the suckling period

Preceding experiments identified SPI-2 as a negotiable factor concerning SCV formation in the neonate small intestine (Figure 6). So far, we only investigated one particular time point and next addressed the question, whether SCVs could not only be established, but also maintained by bacteria lacking modulation strategies ensured by SPI-2 effector proteins (Figueira and Holden 2012).

Within 12 days after oral application of the ΔSPI-2 mutant, the number of SCVs inside intestinal enterocytes of neonatally infected mice remained relatively constant (Figure 11 A). Simultaneously, the bacterial vacuoles continuously increased in size (Figure 11 B+C). Between 12 and 14 dpi, a drop in both number and size of mutant SCVs was observed. Immunofluorescent microscopy pictures suggest an increasing number of bacteria per vacuole during host maturation (Figure 11 C). This hints at intracellular replication of SPI-2-deficient STy inside the neonatal enterocytes up to at least 12 dpi.

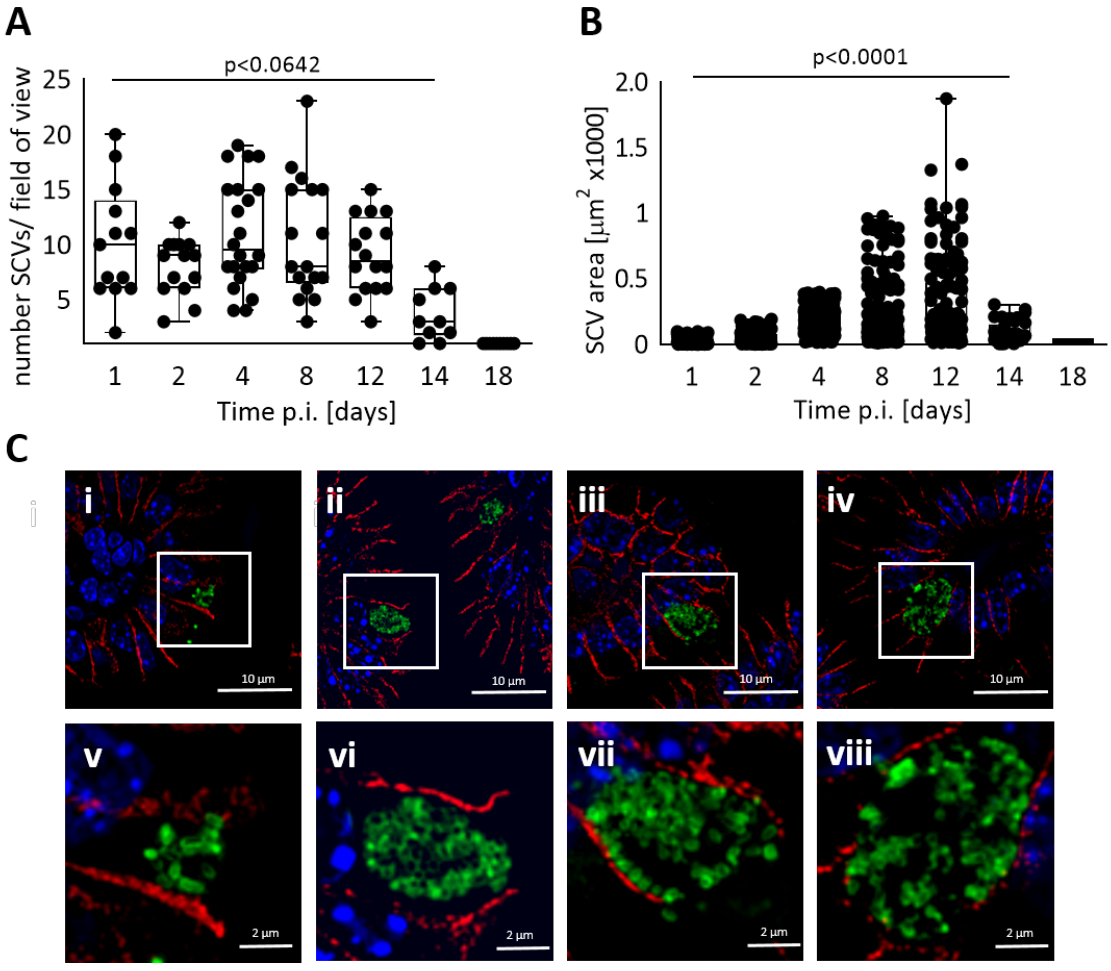

Figure 11: Maintenance of SCVs in the small intestine during the neonate period

One-day old mice were orally infected with 10^2 CFU Δ*sseB Salmonella*. At indicated points of time, mice were sacrificed and the number (A) and size (B) of SCVs during the neonatal period was determined by

immunfluorescence of small intestinal tissue sections. Significance was calculated with ordinary one-way ANOVA. Representative pictures demonstrate an increase in SCV size during the corse of infection with the SPI-2-deficient strain (C). Comparison of intracellular *Salmonella* (green) at 2 dpi (I, v), 4 dpi (ii, vi), 8 dpi (iii, vii) and 12 dpi (iv, viii), vacuoles progressively increase in size, as can be observed in enlarged views (v-viii) of i-iv. Counterstaining of DNA (blue) and E-cadherin (red), scale bars with sizes as indicated.

3.3.2 Metabolic demands of SPI-2-deficient *Salmonella*

Following, we verified intracellular replication in the absence of SPI-2. A prerequisite for replication is the generation of biomass. SPI-2 effector proteins, besides other host cell manipulations, allow the interaction with the host cell`s microtubule network and conversion of the endosomal system into an extensive network of interconnected tubular vesicles, of which *Salmonella*-induced filaments (SIFs) were described to be linked to efficient nutrition inside the host cell (Liss *et al.* 2017). Despite lacking this property, sufficient nutrient supply seems to be feasible in the neonate gut, as indicated by ongoing size increase of mutants' SCVs (Figure 11). Subsequently, we supposed fusion of the SCV with endosomal vesicles of the host cell and conducted an experiment to follow endosomal particles in the next step.

After oral application of gold nanoparticles in BSA/PBS solution at 3 days *p.p.,* their localization was stated within vacuoles in the small intestine, most likely taken up into the host cell via micropinocytosis and fusion of the endosomal vesicle containing nanoparticles with intracellular storage vacuoles, typically seen in the neonatal gut (Figure 12 A-B) (Moon 1972; Skrzypek *et al.* 2007; Staley and Bush 1985). If mice were infected with SPI-2-deficient *Salmonella* prior to nanoparticle application, EM pictures clearly demonstrate co-localization of applied gold particles and mutants` SCVs (Figure 12 C-D).

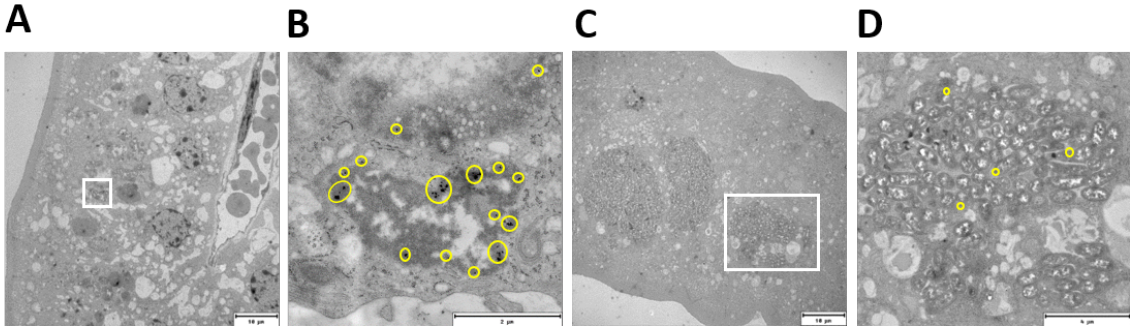

Figure 12: Endosomal fusion with vacuoles and SCVs in the neonatal SI epithelium

One-day old mice were left untreated (A, B) or orally infected with 10^2 CFU Δ*sseB Salmonella* (C, D). At day 4 *p.p.* for the uninfected control animals or 3 dpi for infected animals, respectively, a solution of gold nanoparticles in BSA/PBS was administered orally every 8 hours. Mice were sacrificed 24 hours after first BSA gold administration, small intestinal tracts were removed and prepared for microscopic investigations via TEM (2.2.8). Transmission electron microscopy pictures of small intestinal sections

reveal localization of gold nanoparticles marked by yellow circles (B, D) kindly created by Urska Repnik. Image section in white squares in A and C are enlarged in B and D. Scale bars with sizes as indicated.

These results indicate a fusion of endosomal particles not only with host cell vacuoles (Figure 12 A-B), but with mutants` SCVs as well (Figure 12 C-D). *Salmonella* is a well-known metabolic generalist, that subverts the host`s metabolic pathway for its own proper intracellular nutrition (Dandekar *et al.* 2012; Dandekar *et al.* 2014). Subsequently, we wanted to examine whether certain metabolites are essential for pathogenesis of *Salmonella* or SCV formation and maintenance in the absence of SPI-2.

Hereinafter, we compared the infection of neonatal mice with the "single" ΔSPI-2 strain to double mutants lacking not only a complete T3SS-2, but in addition a functional mechanism for the uptake of peptides, glucose, amino acids or long-chain fatty acids. This comparative approach yielded similar bacterial burden in systemic organs, MLN, intestinal enterocytes and colon (Figure 13 A-E), suggesting that none of the tested metabolites significantly influences SPI-2-independent pathogenesis of STy. Since colonization rates of enterocytes in the small intestine in the absence of certain uptake mechanisms were similar to the ΔSPI-2 mutant strain, the assumption of functional SCV establishment in the double mutants seemed obvious. Indeed, neither the capability of SCV formation, nor their increased size relative to wt *Salmonella* differed in any tested ΔSPI-2 metabolic mutant strain in comparison to the Δ*sseB* strain (Figure 13 F-I). Hence, we concluded that the uptake of peptides, glucose, amino acids or fatty acids is dispensable for *Salmonella* concerning the first steps in pathogenesis and intracellular replication in the neonate host.

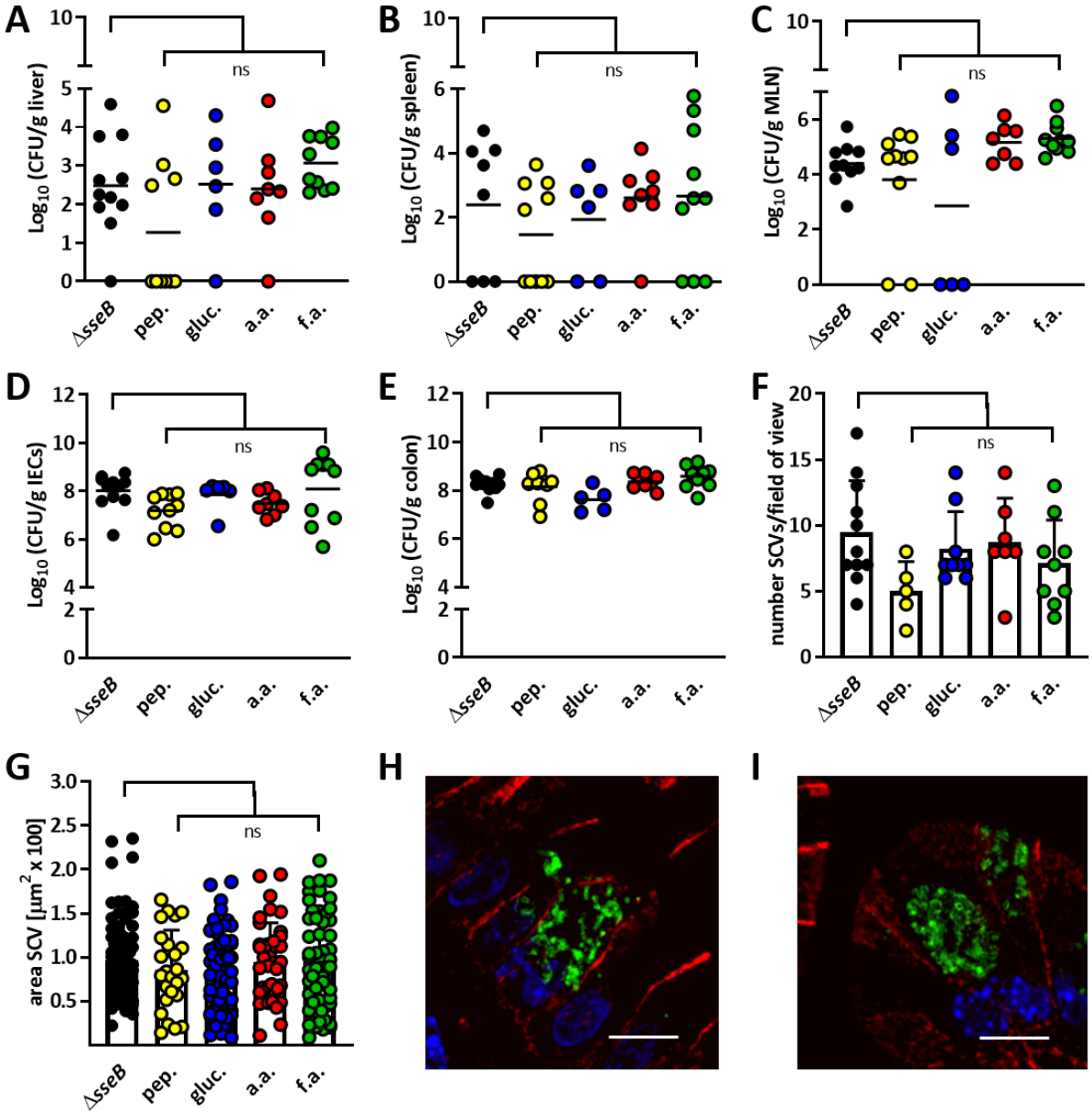

Figure 13: Single metabolic pathways are dispensable in neonatal NTS infections.

One-day old mice were orally infected with 10^2 CFU of either SPI-2-deficient *Salmonella* (Δ*sseB*, black circles), or a ΔSPI-2 mutant strain additionally depleted in the uptake of a certain metabolite as follows: peptides (pep., yellow circles), glucose (gluc., blue circles), amino acids (a.a., red circles) or long-chain fatty acids (f.a., green circles). Mice were sacrificed 4 days p.i. and bacterial dissemination was determinded via replica plating of liver (A), spleen (B), MLN (C), IECs (D) and colon (E). Furthermore, the number (F) and size (G) of mutants' SCVs was investigated in small intestinal tissue sections by immunofluorescence, representitavely shown for the glukose and glucose-6-phosphate (H) and amino acid uptake (I) mutants with *Salmonella* (green), DNA (blue) and E-cadherin (red). ANOVA with Dunn`s post-test, ns = p-value≥0.05.

3.4 Transmigration of polarized enterocytes in the neonatal small intestine by *Salmonella* depends on SPI-2 effector proteins

After the identification of SPI-2 as an expendable factor for intestinal colonization of the neonate gut, SCV development (3.2.1) and bacterial replication inside neonatal enterocytes in the SI (Figure 11), we wanted to pinpoint the function of SPI-2 in neonatal NTS infections.

By histological investigations of wt and Δ*sseB* infected small intestinal tissue sections, we noticed a distinctive localization pattern of wt *Salmonella*, that could not be observed in a mutant infection. Immunofluorescence staining of small intestinal tissue infected with wt STy, capable of SPI-2 effector protein translocation indicated SCVs found in a basolateral position in relation to the nucleus of the host cell (Figure 14 A). This could be further validated by TEM pictures (Figure 14 B). To verify the observations, we quantified the localization of wt SCVs inside the host cell at 2 and 4 dpi (Figure 14 C). Whereas we detected approximately 75% of wt SCVs in an apical position at 2 dpi (white bar), the majority (55 %) of bacterial vacuoles stand basolaterally to the cell nucleus at 4 dpi (black bar) (Figure 14 C). In contrast to this, SCVs formed by STy depleted of SPI-2 were found exclusively at the apical side of infected enterocytes (Figure 14 D) although intact membranes surrounded the huge SCVs of ΔSPI-2 mutant bacteria, shown by TEM (Figure 14 E). Observations of ΔSPI-2 SCVs in terms of cellular localization match immunofluorescence and TEM pictures. Between 2 and 4 dpi, almost 100% of SCVs remained located apically inside enterocytes (white bars) (Figure 14 F). Overall, this proposes a requirement of SPI-2 effectors for transmigration of enterocytes from apical to basolateral, but not intracellular replication.

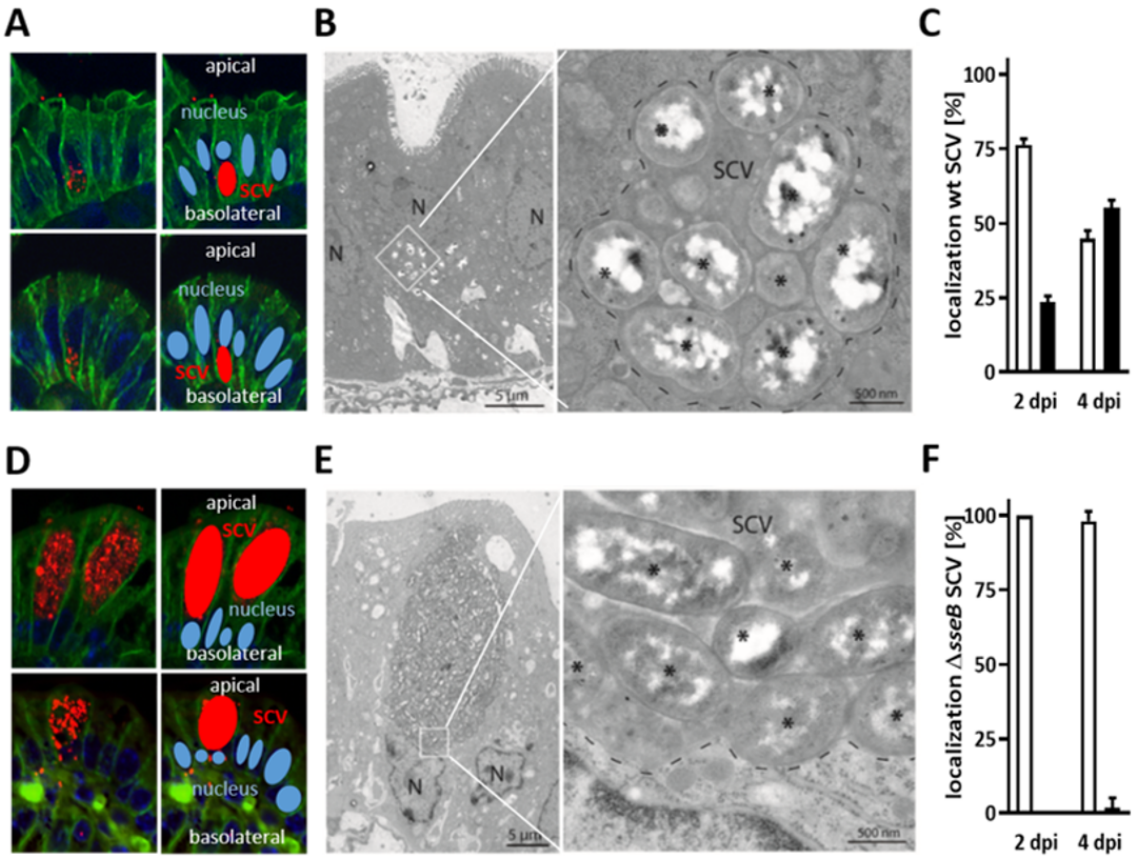

Figure 14: A lack of SPI-2 effectors eradicates SCV transmigration in polarized epithelial cells.

SCVs formed in neonatal enterocytes in STy wt (A-C) and ΔSPI-2 (D-F) challenged mice at 4 dpi, infectious dose 10^2 CFU. Immunofluorescence (wt in A, ΔSPI-2 in D) of infected small intestinal tissue sections with localization of the SCVs (*Salmonella*, red) in relation to the cell nucles (depicted in blue) either at the apical or basolateral side of the polarized epithelial cells. TEM pictures of wt (B) and SPI-2-deficient (E) *Salmonella* in small intestinal tissue of neonatally infected mice at 4 dpi by Urska Repnik. Scale bars indicate given sizes, white boxes indicate zoom-ins depicted in the following picture (tenfold magnification). Broken lines indicate completely membrane enclosed SCVs inside the host cell, stars pinpoint single bacteria, N = nucleus. The SCVs formed by wt and ΔSPI-2 STy were categorized according to their localization to the nucleus as indicated in IF pictures (A+D) at 2 and 4 dpi. White bars indicate the amount of SCVs in an apical position, black bars SCVs basolateral to the nucleus. At least 30 different cells from 3 different animals were evaluated.

3.4.1 Single effectors for interaction with the host cell microtubule network are accountable for the SPI-2-dependent intestinal phenotype

In the current work, we successfully verified functional SCV formation in the neonate small intestinal epithelium for SPI-2-deficient *Salmonella.* However, so far, only mutants depleted of the entire effector protein subset encoded by SPI-2 due to abrogated translocation by the T3SS-2 were investigated and their intestinal phenotype of enlarged SCVs compared to wt *Salmonella* (3.2.1) could not be fully clarified. Hence, we aimed at further identification of specific SPI-2 effectors responsible

for the observed phenotype. We therefore investigated single SPI-2 effector mutants in the neonate host in the following step.

According to their purpose concerning modulation and manipulation of the host cell, effector proteins of SPI-2 can be clustered into different functional groups (Figueira and Holden 2012). Interestingly, those single SPI-2 effector mutants with a significantly decreased bacterial load in systemic organs compared to the wt strain, *ΔsseF/G*, *ΔsifA* and *ΔpipB2*, share the feature of a proven interaction with the host cell`s microtubule system (Figure 15 A-B). Other SPI-2 effector mutants investigated colonized liver and spleen to a similar degree than wt *Salmonella* at 4 dpi. Unlike the complete ΔSPI-2 mutant, ΔsseF/G and ΔpipB2 also exhibited decreased bacterial burden of the small intestinal tract (Figure 15 C). We checked, whether the single SPI-2 mutants did not only resemble the systemic phenotype of ΔSPI-2 *Salmonella* lacking the entire effector protein subset, but also the intestinal phenotype with unaffected SCV formation capability, but increased SCV size. Indeed, quantification of mutants` SCVs at 2 days p.i. revealed similar numbers in infected intestinal tissue sections as detected for wt *Salmonella* (Figure 15 D). Like the Δ*sseB* strain (Figure 6 C), single SPI-2 effector mutants investigated also formed larger SCVs than the wt strain (Figure 15 E), Δ*sseF/G*: p=0.0069, Δ*sifA*: p<0.0001 and Δ*pipB2*: p=0.0237). Summarized, those SPI-2 effector proteins ensuring interaction with microtubules primarily constitute the observed SPI-2-dependent phenotype in the neonate host.

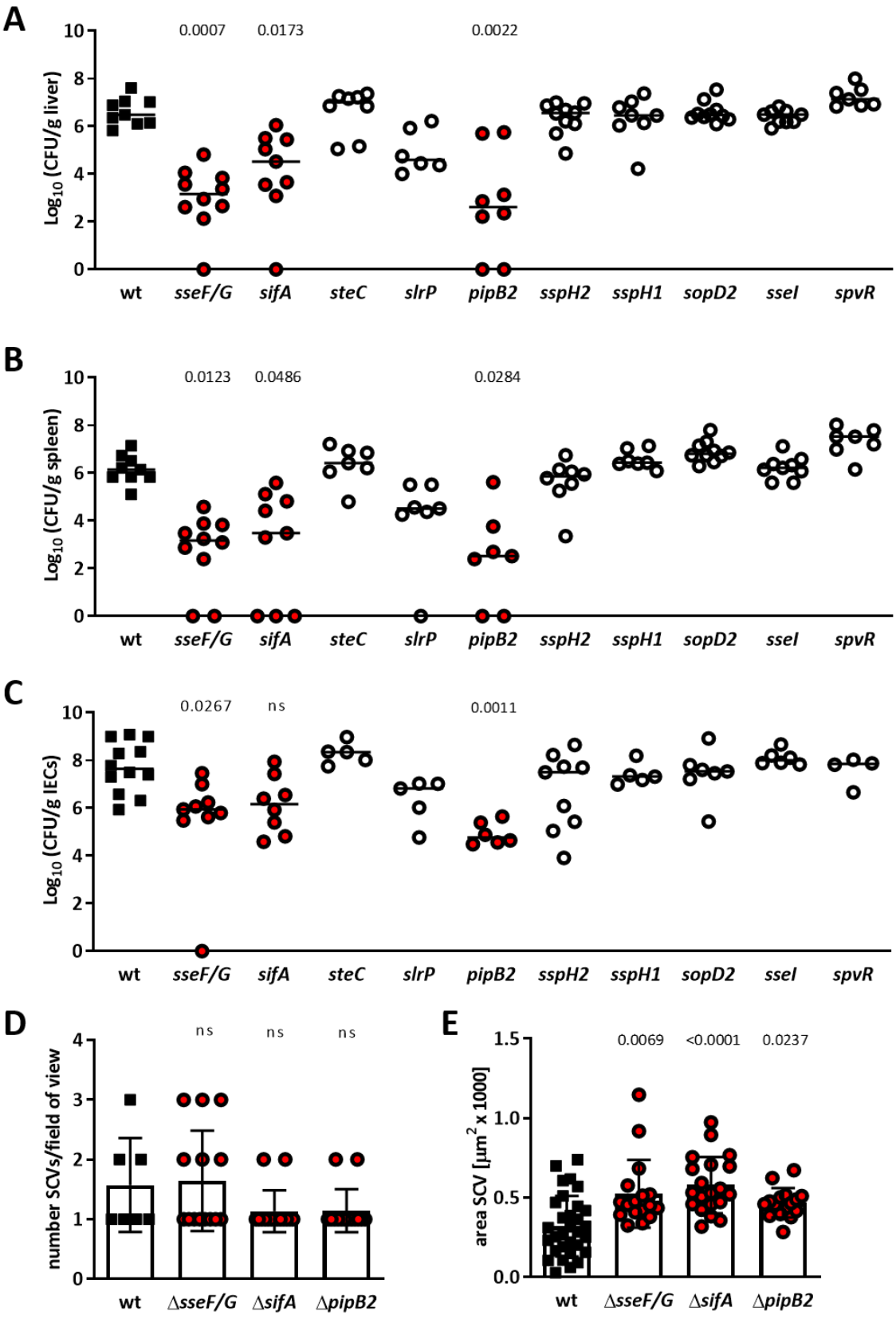

Figure 15: Contribution of individual SPI-2 effector proteins to the observed phenotype

Neonatal mice were orally infected with 10^2 wt *Salmonella* or mutants depleted of the indicated SPI-2 effector protein. Four days p.i., mice were sacrificed and bacterial counts in the liver (A), spleen (B) and small intestine (C) were tested by replica plating. Bacterial strains lacking a SPI-2 effector protein with a proven interaction with the cellular microtubule system of the host are depicted in red. Results

represent median values of 1 to 3 independent experiments. Significance was calculated using ANOVA with Dunn`s multiple post-test. Differences with p≥0.05 are considered not significant and valid if not indicated otherwise. The number (D) and size (E) of SCVs formed by selected mutants (red circles) at 2 days p.i. are represented compared to the wt strain (squares) additionally. Numbers were determined by evaluation of 20 fields of view from 3 different mice. SCV size was determined by measuring 40 SCVs from 3 distinct animals. Results represent median values, significance was calculated by ANOVA with Dunn`s multiple post-test. Differences to the wt strain were not significant unless indicated by the given p-value.

Overall, Δ*sseF/G*, Δ*sifA* and Δ*pipB2* mutant STy resemble the complete SPI-2-deficient phenotype in terms of reduced systemic dissemination (Figure 15 A+B), equal colonization of the intestinal tract as wt STy (Figure 15 C) as well as SCV formation and intravacuolar replication capability (Figure 15 D+E). These results suggest the investigated intestinal SPI-2-dependent phenotype to rely on single SPI-2 effector proteins involved in microtubule interaction, SCV positioning and host cell transmigration. We finally checked, whether a combination of single effectors with an apparent phenotype would result in abrogation of enterocyte transmigration and systemic spread of mutants in the neonate. In the next step, we investigated the intestinal colonization and systemic dissemination of two multi SPI-2 effector mutant combinations that contribute to SCV localization and microtubule interaction (Figueira and Holden 2012). Similar to single SPI-2 mutants and the Δ*sseB* strain, a combination of distinct effectors from the same functional group resulted in decreased systemic spread compared to wt bacteria, but equal colonization of the gut of infected neonates.

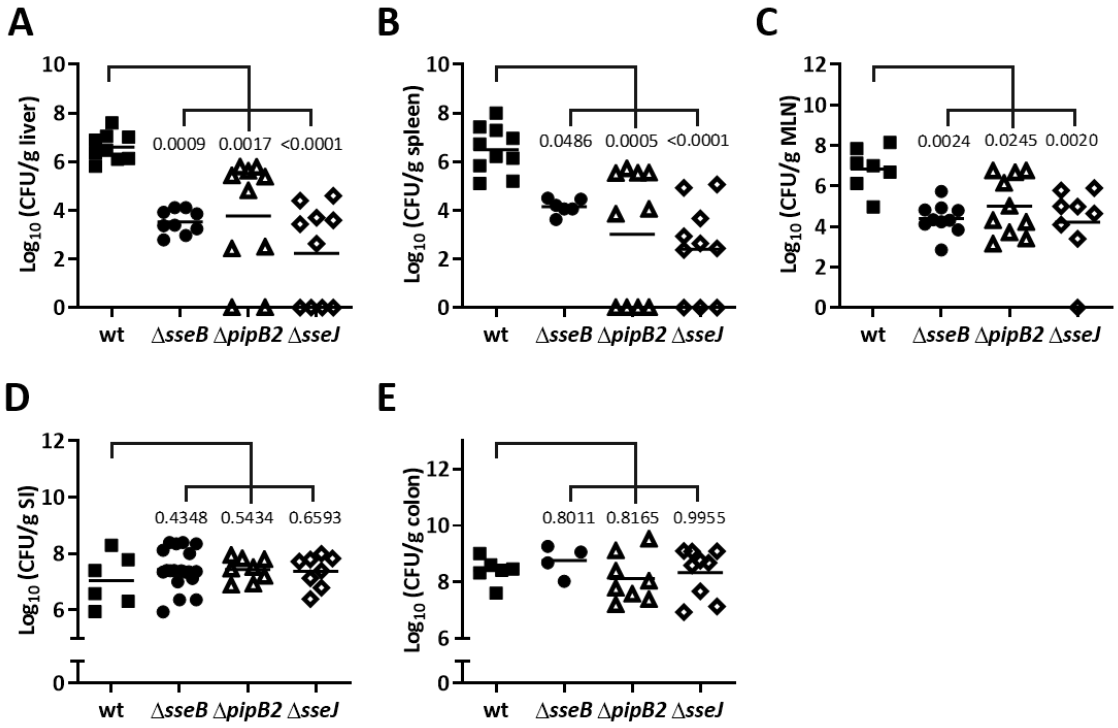

Figure 16: Combined SPI-2 effector mutants resemble the Δ*sseB* phenotype in neonates

One-day old neonate mice were orally challenged with 10^2 CFU multi SPI-2 effector mutant strains and compared to bacterial numbers in STy wt (solid squares) and complete SPI-2-deficient mutants

(circles). Multiple SPI-2 effector-deficient strains were both depleted of *sifA, sseF/G* and the corresponding chaperone *sscB* plus either *pipB2* (labeled *ΔpipB2*, open triangles) or *sseJ* (labeled *ΔsseJ*, open rhombs). Animals were sacrificed at 4 dpi and investigated for bacterial number in total liver (A), spleen (B), MLN (C), IECs (D) and colon (E). Results indicate individual animals from one litter plus the median. Statistical differences were calculated with ANOVA and Dunn's post test.

4. Discussion

The current study focusses on characterizing functions of *Salmonella* pathogenicity island-2 (SPI-2) in disseminative, non-typhoidal *Salmonella* infections of the neonate host. We applied a previously established oral infection model for neonate mice (Zhang *et al.* 2014) combined with a mutant possessing a genetic defect, rendering bacteria unable in assembling the type three-secretion system 2. Deletion of the SPI-2 effector protein subset results in an avirulent infection of the neonate with decreased systemic spread (Figure 3), as has been shown for adult mice (Hensel *et al.* 1995). However, SPI-2 mutants successfully established and maintained SCVs inside small intestinal enterocytes (Figure 6) clearly exhibiting intracellular replication (Figure 11) independent of MYD88-signaling (Figure 9). With similar colonization of the gut, SCVs formed by SPI-2 mutants were even significantly bigger in size than wt SCVs. This led us to the conclusion that SPI-2 effectors seem to be dispensable for intracellular replication and SCV formation in enterocytes of the neonatal small intestine. Certain SPI-2 effectors allow the interaction with the host cell microtubule network. In the absence of these modulators, like e.g. SifA, transmigration of enterocytes seems to be hampered, yielding to a "dead-end" for mutant bacteria within the SCVs, which finally prevents systemic disease.

4.1 Functions of SPI-2 in the neonate host

4.1.1 SPI-2 is crucial for systemic spread in the neonate host

Attenuation of virulence for SPI-2-deficient *Salmonella* in the neonate mouse model is in line with the well accepted role of SPI-2 in intracellular survival (Gerlach and Hensel 2007) and systemic dissemination after oral infection in adult animals (Watson and Holden 2010). Consistent with the latter finding, bacterial burdens observed in this study in systemic organs and the MLN of mutants were markedly reduced compared to wt bacteria (Figure 3). In the adult gastroenteritis model, antibiotic treatment prior to oral *Salmonella* infection and a high infectious dose of 10^8 – 10^9 CFU is required for effective NTS infection (Barthel *et al*. 2003; Miki *et al*. 2017). Competition of the pathogen with the enteric microbiota is omitted in the neonate gut, which is only poorly colonized at the time point of oral challenge, explaining the sufficiency of a lower number of bacteria (10^2 CFU, 2.2.4). In general, it is commonly accepted that SPI-2 is of crucial importance in terms of replication in macrophages at systemic sites and that, in contrast to a ΔSPI-1 mutant, a SPI-2-deficient strain is avirulent in mouse models after intraperitoneal injection (Hensel *et al.* 1995; Shea *et al.* 1996; Ochman *et al.* 1996). A study by Grant and colleagues demonstrated the number of SPI-2-depleted bacteria per phagocyte to be higher than for wt STy, but that mutants displayed deficiencies in cell-to-cell spread

and establishment of new infection foci due to failures in host cell evasion, significantly contributing to virulence attenuation in the adult murine model (Grant *et al.* 2012).

However, bearing in mind the assumption that successful intracellular replication strongly depends on proper SCV formation by SPI-2 effectors, equal colonization of the intestinal tract by wt and ΔSPI-2 STy is counterintuitive in the first place. To a large extent, this is attributable to the immature small intestinal epithelium constituting a unique environment, especially concerning metabolic demands of the intracellular pathogen (Bumann and Schothorst 2017; Noster *et al.* 2019) discussed later in this study (4.3.1). Complementation of *sseB* restores systemic dissemination capability. Compared to wt and SPI-2-deficient bacteria, the number of complemented mutants in intestinal enterocytes was slightly elevated, probably due to the incorporated plasmid for episomal expression (Table 13).

4.1.2 The influence of host maturation on SPI-2-independent NTS pathogenesis

Breaching the epithelial barrier in the intestine is considered the initial step in pathogenesis of NTS-infections (Muller *et al.* 2012). This might be facilitated by different mechanisms, classified into either direct, one-step, or two-step invasion strategies. Intestinal cryptopatches and isolated lymphoid follicles (ILFs), crucial for the intestinal immune system are formed after birth in mice. Specialized epithelial cells, known as microfold (M) cells, reside above ILFs and Peyer's patches and facilitate antigen transport from the lumen to the underlying lymphoid cells (Renz, Brandtzaeg, and Hornef 2012). The invasion of Peyer`s patches via specialized M cells constitutes a potent entry port for *Salmonella* in a two-step process (Hase *et al.* 2009; Santos and Baumler 2004). In adult mouse NTS infections, the most distal Peyer`s patches in the terminal ileum are the primary site of intestinal invasion (Carter and Collins 1974; Jones, Ghori, and Falkow 1994). Since differences between proximal and distal small intestine as well as between enterocytes isolated from the two distinct gut compartments became more apparent with ageing of the host, findings from previous studies are supported (Figure 4).

Zhang and colleagues demonstrated the invasion pathway of *Salmonella* in mice to be highly age dependent. Incomplete M cell development in the neonate host forces bacteria to trigger SPI-1-induced enterocyte invasion and utilize an alternative two-step strategy by invading the absorptive mucosa, whereas fully mature M cells constitute the primary entry port in older animals (Zhang *et al.* 2014). Hence, a probable explanation for bacterial burden in small intestinal tissue and isolated enterocytes of juvenile mice is the onward appearance of functional M cells during host maturation (Figure 4 D-G). Nevertheless and in contrast to neonate mice, invasion of epithelial cells and SCV formation was not observed if animals were infected at a later age and SPI-2-independent

SCV formation only seems feasible at an early neonatal "window of opportunity" and in case *Salmonella* ends up in enterocytes (Renz *et al.* 2018). M cell frequency gradually increases along the proximal-distal axis of the small intestine, peaking in the terminal ileum (Pabst *et al.* 2005). This explicates the observation of more pronounced differences in bacterial loads between proximal and distal SI in older animals that could not be observed in neonatal mice (Figure 4 E+G). In addition to physiological increase in M cells, *Salmonella* triggers differentiation of enterocytes into M cells by the SPI-1 effector protein SopB (Rouch *et al.* 2016; Tahoun *et al.* 2012).

Furthermore, a number of fundamental differences distinguish the neonate and adult gut epithelium in mice, e.g. in terms of the thickness of the protective mucus layer. In adult mice, mucosal translocation of *Salmonella* is strongly affected by the thickness and composition of the intestinal mucus (Zarepour *et al.* 2013; Zhang *et al.* 2014). Paneth cells, secreting a huge range of antimicrobial peptides in the intestinal crypts, are immature in newborn mice. This alters the expression profile during host maturation from a striking secretion of e.g. CRAMP in the neonatal period to a broader defensive peptide spectrum in the adult mouse (Menard *et al.* 2008). This shift in secreted antimicrobial peptides and the mature mucus layer form a defensive shield in adult mice, conferring protection against invading pathogens (Ayabe *et al.* 2000; Dupont *et al.* 2015; Salzman *et al.* 2003; Wilson *et al.* 1999). Finally, exfoliation of enterocytes, that participates in the constant renewal of the intestinal epithelium, is not yet established in newborn mice, revealing a major reason for gastroenteritis caused by NTS being usually self-limiting in healthy adults (de Santa Barbara, van den Brink, and Roberts 2003; Muncan *et al.* 2011).

4.1.3 Intestinal inflammation is induced even in the absence of SPI-2 effectors

Besides the occurrence of mature M cells at 10 to 12 days *p.p.* (de Santa Barbara, van den Brink, and Roberts 2003; Harper *et al.* 2011), colonization resistance enabled by the progressively domiciling microbiota impedes intestinal invasion by *Salmonella*. In line with literature, initial enterocyte invasion by SPI-1 effectors induces a local inflammatory response within the first 6-8 hours after infection (Altmeyer *et al.* 2010; Bruno *et al.* 2009; Patel and Galan 2005) even in the absence of SPI-2, but this induction is only temporal and strikingly diminished compared to virulent wt bacteria at 4 days p.i. (Figure 5). SPI-1-induced activation of mitogen-activated protein kinase (MAPK) pathways leads to an enhancement in proinflammatory cytokine production, like e.g. interleukin-8, recruitment of polymorphonuclear leukocytes (PMNs) and induction of acute intestinal inflammation and macrophage cell death, further exacerbating inflammation (Bruno *et al.* 2009; Galyov *et al.* 1997; Lee *et al.* 2000; van der Velden *et al.* 2000). Gut inflammation caused by penetration of the intestinal

barrier by *Salmonella* was identified as a "double-edged sword" being detrimental and beneficial for pathogenesis simultaneously. Antimicrobial substances and reactive oxygen species diminish luminal bacteria on the one hand, but foster transmission and bacterial dissemination via the inflammatory response on the other hand (Maier *et al.* 2014; Santos *et al.* 2009). Exploiting the inflammation enables *Salmonella* to outcompete the inhabitant microbiota by utilizing tetrathionate as an electron acceptor, conferring a highly beneficial growth advantage in the lumen of the inflamed gut (Faber *et al.* 2017; Stecher *et al.* 2007; Winter *et al.* 2010). Inducing inflammation by intestinal invasion might provide benefits for *Salmonella* in terms of competition with other microbes in older mice rather than in neonates, explaining bacterial load of intestinal tissue in mice infected at later time points. However, the capability of SPI-2-independent SCV formation in intestinal epithelial cells seems to be limited to the neonatal period, as no SCVs were detected in mice infected at later stages. Furthermore, migration from the gut to the MLN is impaired in mice infected at 6 and 11 day *p.p.* and no mutants were detected in neither liver nor spleen of these animals (Figure 4 A-C). This leads to the assumption of a SPI-2-independent disseminative pathway from lymphoid organs to systemic compartments that is solely restricted to the neonatal period.

4.2 SPI-2-deficient bacteria display an unexpected phenotype in the neonate intestine

Previous studies identified SPI-2 as an indispensable requirement for SCV formation and hence effective bacterial growth and replication inside the host (Abrahams, Muller, and Hensel 2006). Nevertheless, SPI-2-independent replication in the cytosol of HeLa cells represents an alternative, but comparably slow duplication strategy observed *in vitro* (Malik-Kale, Winfree, and Steele-Mortimer 2012). Other studies demonstrated *Salmonella* ΔSPI-2 mutants to be defective for replication in the cytosol of fibroblasts or macrophages (Beuzon, Salcedo, and Holden 2002). Indeed, to the best of our knowledge, only one particular study claims SPI-2-independent survival and replication of *Salmonella* Typhi in human macrophages (Forest *et al.* 2010). As SPI-2 was observed to be indispensable for replication in various cell lines *in vitro* in the majority of previous examinations (Ochman *et al.* 1996; Helaine *et al.* 2010), the assumption of defective replication of mutant *Salmonella* inside the host animal seems obvious (Cirillo *et al.* 1998; Hensel *et al.* 1998; Shea *et al.* 1999). This is underlined by studies revealing survival of ΔSPI-2 mutants in liver and spleen tissue, but limited to no detectable net growth in numbers (Salcedo *et al.* 2001; Shea *et al.* 1999). In line with this, SPI-2-independent SCV formation in epithelial cells, as far as we know, has never been observed before (Malik-Kale, Winfree, and Steele-Mortimer 2012).

Considering this well-accepted dogma, examination of unimpaired SCV formation potential in the absence of SPI-2 is quite astonishing (Figure 6). Bearing in mind replication defects of mutants lacking the effector proteins of SPI-2 described previously in certain cell lines, this does not seem to be applicable in the intestinal epithelium of neonate mice. SCVs formed in the absence of SPI-2 were even significantly bigger in size than those formed by wt bacteria with intact SPI-2 T3SS effector translocation. High intracellular growth rates of SPI-2-deficient *Salmonella* have been described after intravenous infection of adult mice in $CD18^{+}$ phagocytes before (Grant *et al.* 2012). In addition to high intracellular bacterial densities of the mutants, hampered cell evasion and bacterial spread compared to wt bacteria was monitored. Cytotoxic effects accompanied by functions of SPI-2 effectors proteins, like e.g. SseL and SpvB, result in host cell death and hence have been demonstrated to influence the number of intracellular bacteria *in vitro* (Guiney and Fierer 2011; Rytkonen *et al.* 2007). Nevertheless, single effector mutants did not recreate heavily infected cell characteristics of the SPI-2 mutant. This includes reduced spread and increased number of mutants per cell *in vivo* observed in this and other studies (Grant *et al.* 2012). Findings in this neonatal mouse infection model match previous observations. However, in contrast to the study by Grant and colleagues, in which breaching the intestinal epithelial barrier was bypassed by i.v. injection, infection of neonate mice emphasized the urge of SPI-2 effectors to earlier phases of an infection during intestinal growth. Rather than due to hampered induction of apoptosis, higher bacterial densities of the mutant resulted from reduced cell-to-cell spread. In the absence of a functional NADPH oxidase, the number of infection foci of SPI-2-deficient mutants was similar to those in mice effectively producing ROS as a mechanism of innate host defense (Mastroeni *et al.* 2000; Vazquez-Torres, Jones-Carson, *et al.* 2000). Even though phagocytic NADPH activity might explain diminished systemic spread beyond the gut at least in part, the high intracellular densities and SCV formation of the SPI-2-deficient mutant in the neonate intestinal epithelium as examined in this study remains unclear.

In agreement with the attenuated systemic phenotype, ΔSPI-2 *Salmonella* neither affect the brush border integrity of SCV-containing enterocytes in the small intestine of neonatal mice (Figure 6 D), nor induced any detectable tissue damage.

4.2.1 Population dynamics of NTS pathogenesis and its dependence on SPI-2 effectors

Co-infections with tagged bacteria offer insights into the dynamics of bacterial populations during the course of an infection clearly exceeding possibilities facilitated by “classical” infection experiments (Crimmins and Isberg 2012; Grant *et al.* 2008; Kaiser *et al.* 2013; Kaiser *et al.* 2014; Mastroeni and Grant 2011). Hence, WITS experiments constitute promising opportunities to gain deeper perceptions

into host-pathogen interactions, e.g. in therapeutic aspects and vaccine design (Lim *et al.* 2014; Martinoli, Chiavelli, and Rescigno 2007; Mastroeni *et al.* 2009; Michetti *et al.* 1992; Pabst 2012). A study by Lim and colleagues successfully identified independent colonization of the Peyer`s patches in the intestine, the lymphoid tissue and systemic sites, as well as bottlenecks during the infectious process imposed by the host organism in adult STy-infected mice (Lim *et al.* 2014). Furthermore, distinct cells of the innate immune system, decoyed to the *lamina propria* in the intestine by *Salmonella*-induced inflammation, were shown to be hijacked by the pathogen, carrying bacteria from the gut to systemic organs as a "Trojan horse", thereby enabling systemic dissemination (Maier *et al.* 2014).

Like in other studies, contribution of WITS to the initial inoculum was not equal, but since the number of reads was multiplied with a calculated factor for final evaluation, the imbalance in WITS contribution to the inocula is counterbalanced and can be neglected (2.2.4). As holds true for the adult host, colonization of the neonatal intestine follows non-Darwinian selection processes with identical biological fitness and invasion capacities of each of the tested WITS (Figure 7). Obviously, spread of wt and SPI-2-deficient *Salmonella* follows entirely distinct patterns and processes, as demonstrated by high similarities among all investigated organ compartments in wt but not ΔSPI-2 infected neonates. Considering these findings, a disseminative route of wt bacteria, starting with SPI-1-facilitated intestinal enterocyte invasion, SCV formation and intracellular replication, followed by cell evasion and access to immune cells in the *lamina propria*, spread to the MLN and, finally, systemic dissemination, accompanied by bacteremia and multi organ failure, seems very likely. Analysis of the SI content, especially as compared to the SPI-2 mutant tagged strains infection, revealed unequal distribution of isolated tagged strains and an overall reduction in the number of different WITS in the wt background. Maier and colleagues already described this effect beforehand. In adult STy WITS infected mice, they observed a bottleneck limiting the diversity of the gut luminal pathogen population between day 1-4 p.i. and a drop in cecal and fecal population densities at day 2 p.i. (Maier *et al.* 2014; Stecher *et al.* 2007). On the one hand, this is partly explainable by the inflammatory response induced by the pathogen and, besides this, a phenomenon referred to as "blooming", since the inflamed environment of the gut favors growth of STy in contrast to other bacteria. Nevertheless, even in adult mice harboring a mature adaptive immune system additive to the innate inflammatory response, the gut luminal pathogen population is only temporarily restricted by inflammation, because STy established strategies allowing subversion of the gut luminal inflammation and efficient colonization of this niche (Miki and Hardt 2013; Stecher *et al.* 2007).

However, in the absence of SPI-2, population dynamics utilizing WITS in the Δ*sseB* background showed low similarities to the wt background WITS traced route of infection. A high proportion of different

tagged strains was isolated from the SI content, underlining previous observations and suggesting little to no selection in the intestinal lumen (Lim *et al.* 2014). To a certain extent, this is probably attributable to the diminished inflammatory response in the mutant infection compared to the wt (Figure 5) (Medzhitov 2007). Despite lacking functional SPI-2 effector translocation and the possibility to interact with the host cell`s microtubule network and modify endosomal trafficking by the aid of SPI-2 virulence factors, distribution of tags within SI content and the MLN of mutant infected neonates displayed many overlaps (Figure 7 B, Figure 8 D). This seems contradictory in the first place and demonstrates a SPI-2-independent migration pathway from the gut to lymphatics. On the other hand, infection of systemic tissue seems to originate from a different, unrelated pool of bacteria, as already observed for wt infection of adult mice using the streptomycin model (Lim *et al.* 2014; Maier *et al.* 2014). *Salmonella* Typhi is known to reach the gallbladder and reside in there, which could be applicable for STy as well (Ong *et al.* 2013; Zhang, Hornef, and Fulde 2015). Hence, the detected pool of WITS in systemic compartments might arise from the bile ducts, where, like in the intestine, colonization resistance by residing commensals is established postnatally and was probably not completed at 1 day *p.p.* (Fremont-Rahl *et al.* 2013; Jee *et al.* 2017). Reduced inflammatory host response caused by ΔSPI-2 STy infection abolished bottleneck formation in the gut, in contrast to the wt infection, which elicited apparent histopathological damage (Figure 5) (Hapfelmeier *et al.* 2005). These results reflect literature knowledge, e.g. that the degree of inflammation fuels WITS-diversity loss and the suggestion that the grade of inflammation dictates the efficiency of the intestinal epithelial barrier at least in part (Ackermann *et al.* 2008; Diard *et al.* 2013; Ilg *et al.* 2009; Stecher *et al.* 2008). The key role of inflammation in bottleneck imposition and maintenance of barrier integrity is further supported by transmission of disseminative features to the SPI-2 mutant tagged strains in mixed infection experiments with wt background WITS (Maier *et al.* 2014). The observed reduction in luminal WITS diversity in wt infected adult mice gained in other studies as well as in neonates in this study was ascribed to granulocytes and probably ROS-mediated killing mechanisms previously (Maier *et al.* 2014). Tissue infiltration and transmigration of granulocytes into the gut lumen constitutes a hallmark of *Salmonella*-induced inflammation, hence this bottleneck is pronouncedly examined in virulent WITS infection, but not in the ΔSPI-2 background (Kaiser *et al.* 2012). Still, dissemination to the MLN does not seem to be impaired in the absence of SPI-2 and might be facilitated via DC-mediated transport of *Salmonella*, as suggested earlier (Voedisch *et al.* 2009). DCs constitute an innate immune cell population beneath the epithelial barrier already present at birth in mice (Torow *et al.* 2015). Consistent with this assumption, luminal sampling of *Salmonella* within the intestine has been proposed as a strategy to overcome the epithelial barrier without SPI-1-mediated cellular invasion (Sansonetti 2002).

The population similarities within wt infected liver and spleen compared to the SI content most probably originate from a disseminative route of bacteria as described before. In more detail, the populations isolated from systemic tissue might be the result of accumulative individual, autonomous processes: first, bacteria might reach the liver via the bile duct. Second, DCs sample gut luminal bacteria or a paracellular pathway is utilized in terms of a "leaky gut" and pending gut closure allows spread to the MLN and systemic organs independent of SPI-2 and without intestinal enterocyte invasion (Vukavic 1984; Walker 1979). Third, bacteria invade the epithelial layer in the SI, replicate within SCVs, evade the host cell and spread systemically in a continuous, self-accelerated process, reinforced by massive tissue destruction and immune cell infiltration to the *lamina propria*. Alternatively, single vacuolated bacteria might traverse the epithelial layer without replication inside SCVs in the gut in a SPI-2 independent manner. These processes lead to assimilation of the systemic population to WITS detected in the intestine. As this population alignment is absent in WITS harboring a SPI-2-deficient background, the third pathway is considered the major one for NTS in the neonate host. Synergy of different processes sums up to dissemination to systemic organs, supported by the finding of an accumulation of bacteria in systemic tissue of wt, but not ΔSPI-2 tagged STy between 2 and 4 dpi (Figure 8 E-H).

4.2.2 Functions of MYD88-signaling in NTS pathogenesis in the neonate

The mammalian host poses various challenges to an invading pathogen, due to its complex nature and distinct environments the pathogen is exposed to during the course of infection. As expression of virulence factors is highly costly, the extracellular environment is commonly used by a large variety of pathogens to regulate their expression profile and rapidly respond to environmental changes (Brown *et al.* 2005). Various signals have been implicated in the transcriptional induction of intracellular SPI-2 gene expression in other studies (Chakravortty *et al.* 2005; Deiwick and Hensel 1999). Acidification of the phagolysosome belongs to the most prominent host cell stimuli required for functional SPI-2 effector transcription and translocation (Cirillo *et al.* 1998; Deiwick *et al.* 1999; Deiwick and Hensel 1999; Rappl, Deiwick, and Hensel 2003). In accordance to this, rapid acidification by TLR-signaling has been identified to be crucial for intracellular survival, replication and SCV formation of *Salmonella* in BMMs beforehand (Arpaia *et al.* 2011). Failure to decrease pH inside the vacuole leads to insufficient SPI-2 gene induction and hampers intracellular replication of the pathogen in macrophages isolated from TLR-deficient mice. More globally, despite replication deficiencies in BMMs lacking TLR2, TLR4 and TLR9, KO animals were examined to be less susceptible to STy wt infection, because the pathogen was unable to induce sufficient SPI-2 gene expression in the absence of TLR-signaling (Arpaia *et al.* 2011). Indeed, T3SS-2 was dispensable in the absence of TLR2, 4 and 9 signaling after systemic administration via intraperitoneal (i.p.) infection of adult animals and wt and SPI-2-deficient bacteria

were isolated in equal amounts from systemic tissue early (Sivick *et al.* 2014). In contrast to this, oral infection mimicking natural NTS infection depends on the SPI-2 T3SS, even in the absence of TLR-signaling (Sivick *et al.* 2014). In the neonate mouse model applied in this study, SPI-2-deficient STy were avirulent after oral infection as well, but unlike investigations by Sivick and colleagues, induction of SPI-2 gene expression was possible in intestinal enterocytes lacking downstream TLR-signaling via MYD88. Furthermore and in contrast to BMMs, replication and SCV formation in the absence of functional SPI-2 effector protein translocation was feasible in MYD88 knockout mice (Figure 9 F-G). Other studies also examined SPI-2 expression without intracellular acidification or even epithelial penetration in the ileal lumen of orally challenged mice (Brown *et al.* 2005). Hence, acidic pH seems to be a potent stimulus rather than crucial necessity to induce SPI-2 gene expression *in vivo*. In the absence of TLR-signaling cascades, other environmental cues are obviously exploited by *Salmonella* to induce SPI-2 gene expression (Sivick *et al.* 2014).

Besides facilitating rapid phagosomal acidification, recognition of *Salmonella* and other invading pathogens via PRRs as a central part of the innate immune system is of crucial importance in neonates. This holds true with special respect to the immature and time consuming adaptive immune response (Woolhouse *et al.* 2002). TLRs recognize a variety of conserved microbial ligands associated with invading pathogens, so-called PAMPs (Janeway 1989; Medzhitov 2007). Recognition of *S.* Typhimurium is primarily mediated by TLR2, TLR4 and TLR5, binding lipoproteins, lipopolysaccharides and flagellin, respectively (Fulde *et al.* 2018; Royle *et al.* 2003; Vazquez-Torres *et al.* 2004). According to literature, mice depleted in TLRs, especially TLR4, display higher susceptibilities to STy infection (Weiss *et al.* 2004). Still, unlike previous studies, intracellular replication and SCV formation in the neonate intestine is obviously feasible in the absence of MYD88-signaling cascades. At least in part, this might be attributable to TRIF-dependent signaling via TLR4 in MYD88 KO mice (Stockinger *et al.* 2014). This can not be excluded by our findings, but still seems rather unlikely considering the fact that SCV formation of the ΔSPI-2 mutant is highly similar in the presence and absence of MYD88 (Figure 9 F-H). Recognition of bacterial ligands by cell surface or endosomal TLRs can initiate signaling cascades via MYD88, ultimately leading to e.g. cytokine, chemokine or antimicrobial peptide production (Newton and Dixit 2012). Bearing in mind initial upregulation of inflammatory cytokines upon enterocyte invasion in the absence of SPI-2 (Figure 5), this study emphasizes the uniqueness of the neonate intestinal epithelium, allowing formation of a replicative compartment in the absence of a huge subset of virulence factors and host cell derived innate immune signaling.

4.2.3 Contribution of functional NRAMP1 to NTS pathogenesis in the presence and absence of SPI-2 effectors

Adult NTS-susceptible C57BL/6 mice, which express the mutant *Nramp1*^{D169} allele, were identified being unable to control acute infection with STy after i.p. or oral inoculation (Loomis *et al.* 2014). As demonstrated earlier, this is rather due to counteraction of NRAMP1 against the ability of *Salmonella* to prevent lysosomal degradation inside the SCV. This allows the normal degradative pathway of macrophages to proceed and eradicate the pathogen in the presence of functional NRAMP1 (Cuellar-Mata *et al.* 2002). Furthermore, NRAMP1 alters the maturation pattern of the SCV in cultured phagocytic cells, like RAW264.7, and can mediate the up-regulation of SPI-2 virulence genes in the murine typhoid model (Zaharik *et al.* 2002). Nevertheless, Zaharik and colleagues described a necessity for SPI-2 effector translocation for functional replication in splenic tissue of *Nramp*$^{+/+}$ mice.

As one would expect considering absence of NRAMP1 in cells of the epithelial barrier lining, effects by possession of functional NRAMP1 of mice only became apparent beyond the gut in systemic tissue and lymphatics, harboring immune cells such as macrophages, neutrophils and DCs expressing the ion channel in their cell membranes (Fritsche *et al.* 2012). Like in the adult host, incorporation of a working *Nramp1* dramatically reduced bacterial burden in systemic tissue in the fully virulent wt *Salmonella* strain infection (Figure 10 A-C). The number of ΔSPI-2 mutants, as compared to the wt strain, was reduced in *Nramp*$^{+}$ neonates, although to a smaller extent than in the absence of NRAMP1-mediated killing (Table 11, Figure 10). However, genetic background of the pathogen had a far greater impact on bacterial loads in systemic organs than the host's genotype. Infection experiments were terminated after 4 days, nevertheless complete protection to STy wt infection in *Nramp*$^{+}$ neonates is rather unlikely. More probable, higher effectivity in killing the pathogen by macrophages and leukocytes only delays pathogenesis but flooding of bacteria from the intestine into the blood stream and systemic organs remains lethal to the immature host. Furthermore, as demonstrated earlier, greater splenomegaly, blood neutrophil and monocyte counts, as well as higher proinflammatory serum cytokine and chemokine responses were investigated in adult mice C57BL/6 *Nramp1*^{G169} with restored NRAMP1 compared to Sv129S6 mice with an intact allele. This suggests that other host factors, besides NRAMP1, are involved in resistance against NTS infection (Brown *et al.* 2013; Loomis *et al.* 2014).

4.3 SCV formation and maintenance in the absence of SPI-2

The major understanding of NTS pathogenesis states that distinct virulence systems operate during the intestinal and systemic phases of infection and that these virulence systems display little overlap in their spatiotemporal activation (Brown *et al.* 2005). Our studies so far demonstrated that SPI-2 is

dispensable in terms of SCV formation and intracellular replication in the neonate intestine (Figure 6). However, during maturation, major changes influence physiology of the intestinal epithelium (Chassin *et al.* 2010; Renz, Brandtzaeg, and Hornef 2012). We decided to investigate consequences of intestinal development on intracellular SPI-2 mutants in the next step and examined mutants` SCVs until weaning. Surprisingly, SCVs are not only stably maintained during the first 12 days after oral challenge of neonates, but furthermore even progressively increase in size (Figure 11). This was completely unexpected, as one major function of SPI-2 effectors is the formation of tubular extensions, called *Salmonella*-induced filaments (SIFs) from the SCV, thereby rendering it a replicative niche by ensuring appropriate nutrient supply (Birmingham *et al.* 2005; Brumell, Tang, *et al.* 2001; Krieger *et al.* 2014). Although this is no longer feasible in the absence of a functional SPI-2 T3SS, mutants preserved their intracellular vacuolar compartment and increased in numbers inside infected enterocytes, most probably due to ongoing bacterial replication. Since the number of SCVs remained relatively stable, but establishment of new SCVs due to luminal re-infection seems very unlikely (3.1.2), the most probable explanation for the observed phenotype is initial SPI-1-mediated enterocyte invasion, SPI-2-independent SCV formation and intracellular replication. Although an initial inflammatory response via e.g. *RegIIIγ* expression could be monitored in the absence of SPI-2, expression adjusted to levels detected in uninfected control mice over time (Figure 5), indicating no harm to the host cell by intracellular SPI-2 mutants and supported by the finding of intact cell membrane and brush border (Figure 6 D). The drop in SCV number between 12 and 14 days p.i. perhaps results from intestinal maturation, involving stem cell development, intestinal crypt formation and cell renewal (Renz, Brandtzaeg, and Hornef 2012). Nascent desquamation leads to the loss of infected epithelial cells and intestinal clearance over time.

4.3.1 Unique metabolic properties of the neonatal intestinal epithelium influence metabolic demands of *Salmonella*

As for very many other pathogens, survival of *Salmonella* highly depends on successful adaptation to the different environmental features that bacteria need to face during pathogenesis. This especially holds true concerning intracellular metabolism and bacterial proliferation inside the host cell. *Salmonella* is known as a metabolic generalist, capable of utilizing a huge variety of distinct substances, including e.g. mono- and disaccharides, fatty acids and peptides (Dandekar *et al.* 2012; Dandekar *et al.* 2014). However, in the current understanding, SPI-2 effectors were crucial for intracellular survival in terms of formation of tubular extensions from the SCV in different host cells. These tubular, membranous extensions from the SCV observed in HeLa cells, termed '*Salmonella*-induced filaments' or SIFs were shown to contain lysosomal membrane glycoproteins, indicative of vesicular fusion and thus might be of particular importance for *Salmonella* concerning intracellular nutrition (Garcia-del

Portillo *et al.* 1993). Additionally bearing in mind the special properties of the neonatal intestine during the suckling period, we wanted to examine intracellular metabolism of *Salmonella* in the absence of SPI-2 in more detail. Increase in SCV size and bacterial numbers per SCV observed in this study is most likely attributable to ongoing bacterial replication, and this generation of new biomass depends on the utilization of certain metabolites and proper nutrition (Figure 12) (Liss *et al.* 2017; Sindhwani *et al.* 2017). Microcolony formation was demonstrated to result from invasion of a single bacterial cell rather than clumps of bacteria as a process of clonal expansion (Grant *et al.* 2012; Zhang *et al.* 2018). Opposing to this, SPI-2-deficient mutant bacteria were isolated from the intestinal tract of orally challenged neonates at a similar rate as wt bacteria, indicating that intracellular nutrition and replication of *Salmonella* is obviously independent of SPI-2 effector protein functions and SIF formation (Figure 3).

Detection of orally applied gold nanoparticles in BSA/PBS solution in uninfected vacuoles as well as SCVs of SPI-2-deficient *Salmonella* gives hints to the assumption of endosomal or lysosomal vesicles fusing with enterocytic vacuoles, as well as membrane-enclosed accumulations of mutants inside the host cell (Figure 12). Nevertheless, intracellular mutant bacteria are not killed and degraded by the host cell, but instead retain their SCV inside enterocytes without harming the host cell for at least 12 days p.i. (3.3.1). This resembles recent findings, demonstrating an accumulation of lysosomal proteins, like e.g. LAMP1, in the SCV without enrichment in lysosomal hydrolases (Domingues, Holden, and Mota 2014; Steele-Mortimer 2008). Intestinal growth, dissemination to systemic organs and establishment of SCVs of mutants defective in SPI-2 effector translocation as well as the uptake of either sugars, peptides, amino acids or long chain fatty acids was almost identical to the behavior and traits of the SPI-2 mutant fully functional in uptake of all mentioned substances (Figure 13). As a consequence, none of these substances seems to be crucial for SCV formation and intracellular nutrition in the neonate host. Usually, attenuated virulence of auxotrophic mutant *Salmonella* is based on defective intracellular replication due to auxotrophies in a murine model of typhoid fever (Fields *et al.* 1986). In these adult animal models, the SCV is commonly considered as a nutritional deprived environment (Mastroeni *et al.* 2009). Functional replication of mutants defective in the uptake of certain common metabolites in the neonate gut matches the commonly accepted understanding of the generalized metabolic properties and demands of *Salmonella* observed in other studies (Bowden *et al.* 2009; Lim *et al.* 2010; Steeb *et al.* 2013). In neonatal NTS infections, *Salmonella* appears to be capable of exploiting the unique features of the intestinal enterocytes for intracellular survival and proliferation.

Overall, findings of these studies assemble to the following presumption: Unique metabolic demands and transport machineries of the neonate intestine favor strong vacuolation of enterocytes and formation of enlarged vacuoles inside the gut in early life of different mammals. Virulence factors encoded by SPI-2 enable *Salmonella* to modulate the host cell system in various ways, including

interactions with the endocytic pathway and manipulation of the endosomal trafficking network (Domingues, Holden, and Mota 2014; Figueira and Holden 2012). A population of so-called foetal-type enterocytes or vacuolated foetal enterocytes (VFE) is solely found in newborns and vanishes as the intestine matures (Moon 1972; Skrzypek *et al.* 2007). Uptake and transfer of macromolecules from the gut lumen across the epithelial barrier responsible for nutrition and acquisition of maternal immunoglobulins is a non-selective process (Palm 2019; Walker and Isselbacher 1974). NTS are taken up or actively invade enterocytes at the apical side, irrespective of possession of SPI-2. In the neonate, *Salmonella* might accidentally end up in giant vacuoles distinctive of VFEs. Even in the absence of effector proteins crucial for virulence in adult and neonate mice and formation of SIFs, host-induced, passive processes seem to facilitate fusion of endosomal vesicles with the SCV. Consequently, intracellular replication and proliferation of *Salmonella* is feasible in the absence of SPI-2 effectors and establishment of an extensive interconnected tubular network in the neonate gut.

4.4 SPI-2 effector proteins function in transmigration of enterocytes in the neonate small intestine instead of SCV formation

Considering SPI-2 to be superfluous in terms of SCV formation, we next considered a passive or host cell-mediated process concerning vacuole formation inside the neonates` intestinal tracts. So far, we identified the phenotype of a SPI-2-deficient *Salmonella* strain as being entirely different from effects observed in macrophages or other isolated cells cultured *in vitro*. We observed the striking contrast of unaffected SCV formation and intracellular bacterial proliferation of the mutant. However, the function of SPI-2 in the neonate gut remains ill defined.

Although the induction and regulation of SIF formation via SPI-2 effectors appears to be crucial in adult animals and mature host cells in general, in terms of intracellular nutrition and proliferation, the neonate gut constitutes a special environment with regard to metabolic properties and biochemical as well as structural characteristics (Noster *et al.* 2019). Besides the immature intestinal barrier in neonate mammals, especially the distal small intestine harbors highly endocytic vacuolated enterocytes (Arevalo Sureda *et al.* 2016). In contrast to adults, macromolecules can be transferred across the SI epithelial barrier by transcellular endocytosis from the gut lumen into the blood circulation (Kumagai *et al.* 2011). Previously mentioned VFEs can be recognized by the possession of large or giant vacuoles, which can be subdivided into two distinct types: giant transport and giant digestive vacuoles (Skrzypek *et al.* 2018). These enterocytes, enabling important functions in transport of maternal immunoglobulins and other molecules across the epithelial barrier, were detected in many mammals, including humans, pigs and rodents such as rats and mice (Dekaney, Bazer, and Jaeger 1997; Kraehenbuhl, Bron, and Sordat 1979; Moxey and Trier 1979; Muncan *et al.* 2011). Transport vacuoles

engulf luminal content via endocytosis, move to the basal part of the enterocyte and release their content via exocytosis into the underlying intercellular space. Digestive vacuoles, in contrast, were predominantly detected above the nucleus in piglets and often occupy up to ⅔ of the enterocyte`s volume (Skrzypek *et al.* 2018). During transition from a milk-digesting to a solid food-adapted network at weaning, morphological changes are accompanied by functional adaptations, leading to gradual disappearance of vacuolated enterocytes and reduced permeability to macromolecules (Remis *et al.* 2014; Walthall *et al.* 2005). This transport of molecules into the blood is non-selective, while biological activity of enterocytes is preserved (Babbar *et al.* 1990; Seifert and Sass 1990). Under these unique circumstances, the neonate intestine probably provides ideal conditions for intracellular nutrition and replication of NTS, irrespective of certain virulence determinants.

4.4.1 ΔSPI-2 SCVs occupy an apical position in neonatal intestinal enterocytes

Upon examination of giant ΔSPI-2 SCVs in the neonate gut, we observed a typical localization in an apical nuclear position, whereas wt SCVs were also detected basolateral to the host cell nucleus (Figure 14). Documentation of the SCV positions during the first 4 days of NTS infection revealed that 55 % of wt bacteria managed to transmigrate the epithelial cell in the neonate intestine. Against this, the overwhelming majority of SPI-2-deficient SCVs remained in an apical position of the infected enterocytes without passing the nucleus throughout the observed period (Figure 14). This is not too surprising, keeping in mind that different effectors of SPI-2 function in membrane dynamics, vesicular trafficking and intracellular positioning of the SCV are missing(Abrahams, Muller, and Hensel 2006; Bakowski, Braun, and Brumell 2008; Figueira and Holden 2012; Ramsden, Holden, and Mota 2007; Schroeder, Mota, and Meresse 2011). In addition, SPI-2-deficient *Salmonella* were shown to have high tendencies to remain in an apical position within infected enterocytes in adult animals before, whereas wt bacteria functionally traversed the epithelium, exited at the basolateral side and gained excess to the *lamina propria* (Muller *et al.* 2012). However, in adult animals, single cells rather than SCVs where detected inside intestinal host cells so far.

4.4.2 Single SPI-2 effector proteins are responsible for the observed phenotype of mutants in the neonatal intestinal epithelium

Effector proteins of SPI-2 enable a versatile variety of functions concerning interferences and alterations of host cell processes. These include maintenance of SCV membrane integrity and its proper localization near the Golgi of host cells, modulations of the host cytoskeleton and restriction of immune signaling (Figueira and Holden 2012). Aiming at the identification of the SPI-2 effector eliciting the investigated phenotype of enlarged SCVs in the neonate intestine, single SPI-2 effector protein

knockout mutants were constructed and applied in the neonate NTS mouse infection model in the next step. The impact of certain SPI-2 effectors on virulence has been investigated in other studies beforehand, especially using adult mice and bovine infection models (Gulig and Doyle 1993; Gulig *et al.* 1993; Matsui *et al.* 2001; Rhen, Virtanen, and Makela 1989; Roudier, Fierer, and Guiney 1992; Tsolis, Townsend, *et al.* 1999). Predominantly, attenuated virulence in NTS infections in a SPI-2 effector mutant was attributed to a deficiency in maintaining vacuolar membrane integrity inside the host cell, leading to SCV disassembly and killing of cytosolic bacteria, e.g. in macrophages (Salcedo *et al.* 2001).

Different features of SPI-2 effector proteins need to be considered when interpreting findings from single mutant infection experiments. First, the precise function of various effector proteins in terms of host cell modulation, manipulation or interference has not been fully unraveled so far and remains partially or completely unclear (Figueira and Holden 2012). Second, certain effectors have overlapping functions or redundancies. Third, single effector proteins attenuate or oppose the activity of others as counter players, for instance shown for the acyltransferase SseJ and SifA (Birmingham *et al.* 2005; Patel *et al.* 2019; Ruiz-Albert *et al.* 2002).

By testing individual effector mutants, we were particularly interested in the identification of effectors that resemble the Δ*sseB* translocon mutant, depleted of translocation of the entire SPI-2 effector protein subset with regard to diminished spread to systemic tissue, but equal colonization of the small intestinal tract as well as SCV formation and maintenance capabilities like wt STy. Interestingly, depletion of the majority of tested effectors had no to little effect on systemic spread compared to wt *Salmonella*, except for Δ*sseF/G*, Δ*sifA* and Δ*pipB2* isolated in decreased amounts from liver and spleen of infected neonates (Figure 15 A-B). Remarkably, all these effector mutants attracted attention by a reduced number in the intestine at 4 days p.i. (Figure 15 C). Other effectors involved in F-actin meshwork assembly and additional modifications of the host cell cytoskeleton, like SteC, SseI and SspH2 (Geddes *et al.* 2005; Miao and Miller 2000; Miao *et al.* 2003; Poh *et al.* 2008; Quezada *et al.* 2009; Worley, Ching, and Heffron 2000), or those involved in ubiquitin modification and interference with host cell inflammatory responses via NF-κB, like SspH1 (Haraga and Miller 2003, 2006; Rohde *et al.* 2007), significantly contribute to attenuated virulence in the neonate host in sum, but single KO mutants displayed no phenotype (Figure 15 A-C). Deletion of the *spvR* operon, which significantly affected virulence in other infection models by facilitating NTS spreading, had no effect on neonate NTS pathogenesis (Figure 15) (Gulig and Doyle 1993; Gulig *et al.* 1993; Roudier, Fierer, and Guiney 1992; Rhen, Virtanen, and Makela 1989; Matsui *et al.* 2001).

A shared feature of single mutants that attracted attention concerning diminished systemic spread in the neonate host after oral infection is a proven interaction with the host cell microtubule network, SIF formation and extension (Abrahams, Muller, and Hensel 2006; Boucrot *et al.* 2003; Diacovich *et al.*

2009; Dumont *et al.* 2010; Henry *et al.* 2006; Knodler and Steele-Mortimer 2005; Kuhle and Hensel 2002; Ohlson *et al.* 2008). On the one hand, a bunch of effectors with an observed phenotype play crucial roles in SCV membrane dynamics and integrity (SifA, PipB2) or the positioning of the SCV near the Golgi (SseF, SseG) (Beuzon *et al.* 2000; Deiwick *et al.* 2006; Stein *et al.* 1996). Quantification of mutant SCVs at 4 days p.i. clearly revealed the necessity of the respective effectors in maintaining SCV membrane integrity, for almost no bacterial vacuoles were detected inside enterocytes. However, foreshortening of the evaluation time point to 2 days p.i. illustrates the function of SseF/G, SifA and PipB2 in SCV membrane stabilization. Mutants lacking these proteins clearly tend to develop bigger SCVs than wt STy inside the neonate gut epithelium (Figure 15 D-E). Summarized, mutants deficient in SseF/G, SifA or PipB2 bear a resemblance to the ΔSPI-2 phenotype with diminished systemic spread and enlarged SCVs inside the SI. The destabilization of the SCV in single effector mutants ultimately leads to membrane rupture and bacteria being released into the host cell cytosol. This brings up the question of how these effectors contribute to pathogenesis in the neonate host. To clarify this, it is important to uncover their general function in host cells first.

Maturation of SCVs in polarized epithelial cells is accompanied by basolateral movement of the vacuoles along host cell microtubules towards the juxtanuclear region. They end up at the microtubule-organizing center (MTOC), a structure found in eukaryotic cells from which microtubules emerge and where Golgi stacks accumulate (Harrison *et al.* 2004; Ramsden, Holden, and Mota 2007). This SCV drive is dependent on SPI-2 effectors evoking recruitment of dynein to the SCV via the interaction of RAB7 and Rap-interacting lysosomal protein (RILP) (Harrison *et al.* 2004; Guignot *et al.* 2004). The maintenance of the specific position of the SCV near the MTOC/Golgi was discussed to facilitate bacterial replication, microcolony formation and endosomal-associated vesicular trafficking via the SPI-2 effector proteins SseF and SseG (Mota *et al.* 2009; Salcedo and Holden 2003). Since an SseF/G double mutant displays an identical phenotype to single mutants, including attenuated virulence and a growth defect in macrophages, both effectors are assumed to contribute to the very same function (Deiwick *et al.* 2006). SseF and SseG are integral membrane proteins that interact and thrive dynesin recruitment to microtubule bundles they form, leading to centripetal movement of the SCV in non-polarized, cultured epithelial cells (Deiwick *et al.* 2006; Kuhle and Hensel 2002; Kuhle, Jackel, and Hensel 2004). Furthermore, they have also been shown to be involved in SIF formation (Kuhle and Hensel 2002). A mutant depleted in either SseF, SseG or both effectors undergoes initial juxtanuclear migration and scatters throughout the cell as single bacteria instead of membrane-surrounded microcolonies or bacterial bundles (Deiwick *et al.* 2006; Ramsden, Holden, and Mota 2007). This seems to be applicable for the neonate mouse model, as mutants were hardly detectable inside SCVs at 4 days p.i. and a slightly reduced amount of bacteria was isolated from the gut, although the initial capability to establish SCVs is preserved in the absence of SseF/G (Figure 15

C-D). As a consequence, Δ*sseF/G* mutants are incapable of maintaining vacuolar integrity and the resulting instable SCV membranes highlight the role of these effectors in pathogenesis.

A prominent example of a SPI-2 effector protein enabling various functions in NTS pathogenesis is SifA. Harboring a caspase-3 cleavage site, the effector is potentially divided into two functional domains with different interaction partners and features (Patel *et al.* 2019; Srikanth *et al.* 2010). Membrane tubules induced by *Salmonella* via translocation of the SPI-2 effector SifA have been discussed as a potential strategy to subvert the host cell endosomal system beforehand. Although the establishment of SIFs is not crucial for nutrition in the neonate enterocyte, SifA mutans gradually lose the vacuolar membrane surrounding them several hours after uptake into host cells, probably affecting pathogenesis in the adult as well as neonate host (Beuzon, Salcedo, and Holden 2002). Highlighting a crucial function of SifA in maintaining SCV membrane integrity, as described for SseF/G in the previous paragraph, mutants lacking this effector are released into the cytosol, which might lead to rapid killing of the pathogen in certain cell types, e.g. macrophages (Beuzon *et al.* 2000; Beuzon, Salcedo, and Holden 2002). Consequently, SifA mutants display a strong attenuation in virulence in adult mouse infections, although no replication defect in epithelial cells was observed *in vitro* (Stein *et al.* 1996). Interestingly, SPI-2 T3SS null mutants lacking SifA and all other SPI-2 effectors retained intact vacuolar membranes, indicating contribution of various effectors to SCV membrane integrity and dynamics (Figure 6, Figure 11, Figure 14) (Beuzon *et al.* 2000). This phenotype observed in other studies was also attained in neonate mouse infections with a *sifA*-deficient mutant (Figure 15). Colonization rates of systemic tissue was reduced compared to the wt, however, the mutant displayed difficulties in maintaining membrane integrity inside host cells at 4 days p.i., which also explains slightly diminished bacterial loads in the gut (Figure 15 C). Like the Δ*sseF/G* mutant, Δ*sifA* bacteria were found in bigger SCVs compared to wt STy at 2 days p.i., similar to the Δ*sseB* strain (Figure 15 E). Bearing in mind that virulence of Δ*sifA Salmonella* is not abolished but delayed in the neonate host (data not shown), SPI-2 effector redundancies are reinforced and other proteins probably still enable systemic spread in the absence of SifA. Delayed virulence of the Δ*sifA* strain might be unraveled considering another important function of the effector protein. By binding of the N-terminal region of SifA to the C-terminal pleckstrin homology domain of the host cell protein SKIP (SifA and kinesin-interacting protein), interaction with the light chain of kinesin is ensured (Diacovich *et al.* 2009; Dumont *et al.* 2010; Jackson *et al.* 2008; Ohlson *et al.* 2008). The motor protein kinesin promotes anterograde transport of vesicles along microtubules and the interaction via utilization of SifA probably also allows SCVs to travel along the microtubule network to the basolateral side of the intestinal enterocyte (Rosa-Ferreira and Munro 2011). Hence, a Δ*sifA* mutant exhibits delayed virulence and decreased systemic loads compared to wt *Salmonella* due to hampered transmigration of enterocytes as one of the first steps in NTS pathogenesis in the neonate. A deficiency in SKIP and microtubule interaction abrogates SCV

transmigration and SifA-deficient bacteria attract attention by increased SCV size at 2 days p.i. (Figure 15 D-E). The destabilization of the SCV membrane in the absence of a crucial SPI-2 effector explains decreased amounts of detected SCVs in the neonate gut at 4 days p.i. and in addition the lower bacterial burden in the SI.

Another important SPI-2 effector protein participating in vacuolar membrane dynamics adding to the function of SifA is PipB2 (Henry *et al.* 2006). It seems to be responsible for kinesin recruitment to the SCV membrane, even in the absence of SifA and its interaction partner SKIP (Boucrot *et al.* 2003). The localization of PipB2 to the SCV membrane has already been revealed, yet the exact function of the effector protein has not been determined so far (Knodler *et al.* 2003). A lack of PipB2 did not alter development of the systemic phase of *Salmonella* infection in adult mouse models, but affected the inflammatory response in bovine infections (Knodler *et al.* 2003; Wood *et al.* 1998). PipB2 recruits kinesin to the SCV membrane and interacts with its light chain, but how it contributes to vacuolar membrane stabilization and the process of SCV rupture in the absence of certain effectors is incompletely understood (Henry *et al.* 2006; Knodler and Steele-Mortimer 2005). Although Δ*pipB2* mutants displayed smaller SCVs than mutants deficient in either SseF/G or SifA at 2 days p.i., vacuoles of Δ*pipB2* STy were bigger than wt SCVs (Figure 15 D-E) and the number of bacteria isolated from liver or spleen was markedly reduced as well (Figure 15 A-B). This emphasizes a substantial contribution of PipB2 to pathogenesis of NTS in the neonate, probably by promoting transmigration potential ensured by the interaction of SifA with the microtubule network via recruitment of kinesin to the SCV membrane. In addition, PipB2 may also stabilize the vacuolar compartment. This fits literature findings, as inhibition of kinesin activity increased membrane stability around Δ*sifA* bacteria, but vacuolar membrane loss was still observed in the absence of PipB2 in a Δ*sifA* Δ*pipB2* double mutant (Guignot *et al.* 2004; Henry *et al.* 2006).

Contribution of different effector proteins and an interplay or counteraction within the same functional group is further evidenced by utilization of multiple effector protein-deficient mutants. Four different proteins were identified to affect SCV membrane dynamics: SifA, PipB2, SopD2 and SseJ, whereas SseF and SseG mainly function in positioning of the vacuole inside the host cell and additionally promote formation of microtubule bundles (Figueira and Holden 2012). Interestingly, *sopD2* deletion did not yield an attenuated phenotype in terms of systemic growth in the neonate mouse model, suggesting that its role in maintenance of vacuolar membrane integrity is minor compared to SifA or PipB2. Two different combinations of effectors have been investigated in this study. In both cases, *sseF/G* and *sifA* were deleted and either *pipB2* (Figure 16, open triangles) or *sseJ* (Figure 16, open rhombs) additionally. Like already observed in single SPI-2 effector mutant infections, the number of bacteria isolated from liver and spleen was diminished compared to the wt STy infection

at 4 days p.i. and approximates the Δ*sseB* mutant, whereas colonization of the intestine was more or less identical among all different strains (Figure 16). Furthermore, no SCVs could be detected for either of the multi SPI-2 effector mutant 4 days after inoculation, accounting for a vigorous membrane destabilization in the absence of these effectors. Anyhow, this holds true for single effector mutants, like e.g. Δ*sifA* STy, and underlines a role in stabilizing the SCV membrane of the proteins previously investigated. Findings from multi mutant infections in the neonate emphasize an interplay between different effectors and indicate an imbalance observable by membrane rupture if effector activity misses a counter player, since the effects can be opposing one another (Ruiz-Albert *et al.* 2002). By deletion of SopD2 in addition to other effectors involved in SCV membrane dynamics and maintenance of vacuolar integrity, the SPI-2 null mutant phenotype might therefore be completely reconstructed, including enlarged SCVs with intact membrane surroundings, abolished transmigration due to absent microtubule network interaction and attenuated virulence in the neonate host.

Summarized, single SPI-2 effector mutants as well as a combination of different effector proteins from one functional group stressed the redundancy of effector functions. Single mutants retained virulence and were rather delayed than abolished in eliciting systemic NTS infections. Furthermore, it could be proven that some virulence factors rely on an interaction partner and that presence of certain effectors was crucial for proper function of another one.

4.5 Model of SPI-2-dependent pathogenesis in the neonate intestine

Summarizing the results achieved during this work, we propose an infection model for the function of SPI-2 in the first steps of pathogenesis of iNTS infection in the neonate host (Figure 17).

For the wt strain, a non-phagocytic cell in the neonate intestinal epithelium is invaded via translocation of SPI-1 effector proteins, leading to reorganization of the actin cytoskeleton, membrane ruffling and bacterial uptake, as demonstrated earlier (Zhang *et al.* 2014). Invasion of the non-phagocytic host cell in a macropinosomal-like fashion results in SCV formation. As a consequence, SPI-2 effector proteins will be synthesized and secreted into the host cell cytosol via the T3SS of SPI-2. Certain effectors, like SifA, allow the interaction with the host cell microtubule network via the host protein SKIP (Dumont *et al.* 2010). This interaction ensures transmigration of the SCV from apical to basolateral. Following, *Salmonella* evades the enterocyte and gains access to the *lamina propria* and residing immune cells. The pathogen spreads throughout the body, thereby causing a life-threatening invasive infection and massive inflammation in the gut (Figure 5, Figure 14, Figure 15, Figure 16).

In contrast to this, the capability to establish a functional T3SS for secretion of SPI-2 effectors is depleted in the investigated Δ*sseB* mutant. Invasion and SCV formation remain functional in the

absence of SPI-2 (Figure 3 D, Figure 6). However, the mutant is unable to assemble a complete needle apparatus for SPI-2 effector protein translocation (Figure 17). As a result, interaction with SKIP via SifA and resulting host cell transmigration is blocked for the mutant. Despite lacking the entire subset of SPI-2 effectors, bacterial replication inside the host cell within SCVs is unaffected (Figure 11). Mutant replication proceeds, but SCVs are stuck in an apical position inside the infected enterocyte, because SPI-2 effector protein enabled transmigration via subversion of the microtubule network is blocked (Figure 11, Figure 14). Although SIF formation via SPI-2 effectors and the resulting modification of the host cell's endosomal system was indispensable in adult mice, the unique nature of endosomal trafficking and non-selective transport of macromolecules through the intestinal barrier allows sufficient nutrient supply for *Salmonella* in the neonate gut. By this means, *Salmonella* might end up in giant vacuoles of VFEs, thereby enabling nutrition and proliferation inside the host cell in the absence of SPI-2 effectors and the resulting modulations. In case of the SPI-2 mutant strain, SCVs inside intestinal enterocytes remain a replicative niche, but a dead-end to the pathogen simultaneously. This explains increased sizes of mutant SCVs compared to the wt strain, diminished systemic loads and attenuated virulence in the neonate host observed in this study.

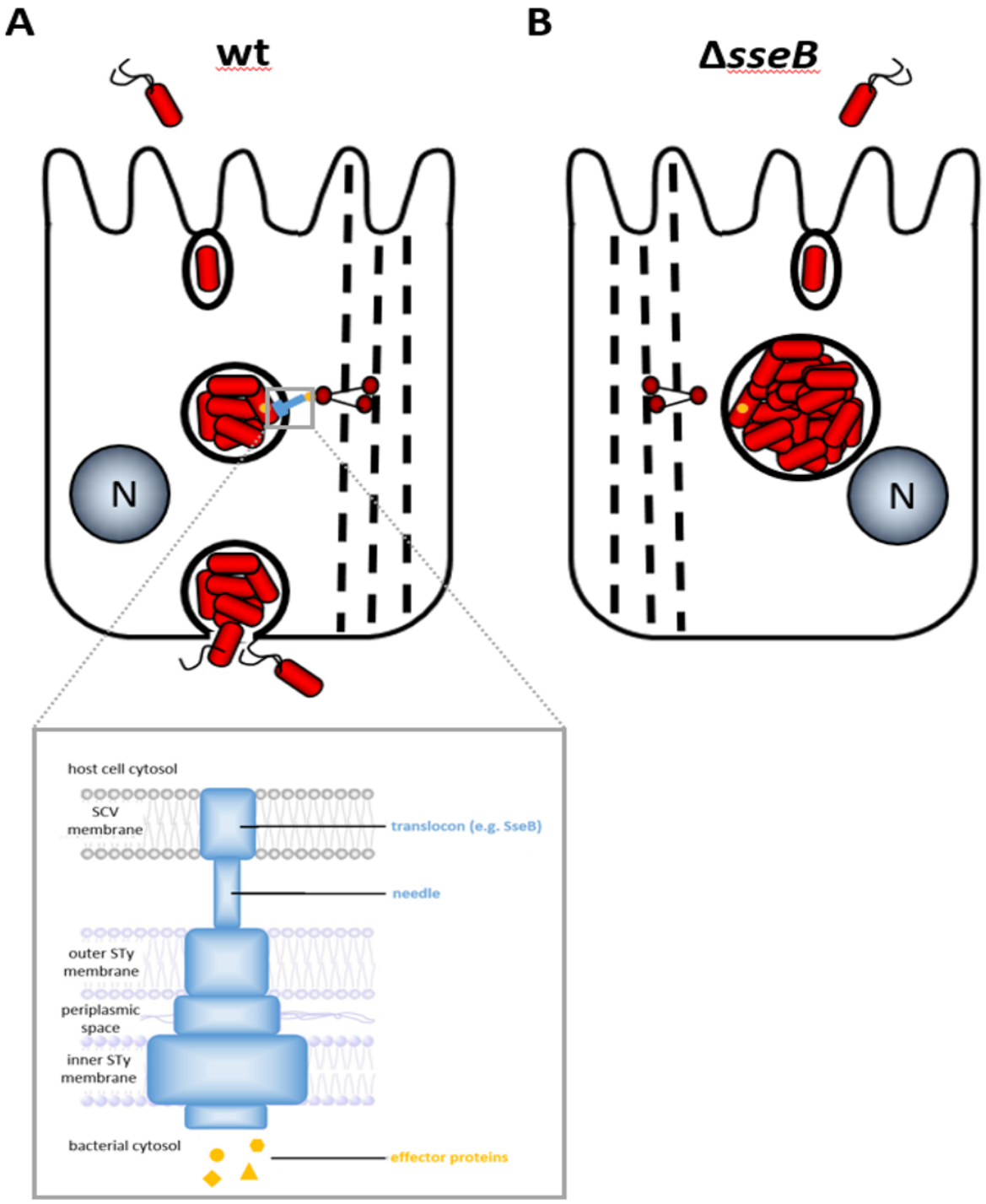

Figure 17: Proposed model of SPI-2-dependent enterocyte transmigration in the neonatal SI

Schemativ comparison of enterocytes in the SI of neonate mice infected with either a wt (A) or SPI-2-deficient *Salmonella*. *Salmonella* possesses SPIs, allowing first enterocyte invasion, followed by translocation of SPI-2 effectors, modulating and manipulating the host cell machinery in various ways, including positioning of the SCV in close proximetry to the nucleus (N). A type three secretion system (T3SS, blue needle structure) allows translocation of SPI-2 effector proteins, like SifA (yellow circle) from the inside of the SCV into the host cell cytosol as can be seen in enlarged view from grey square. The needle-like compartment, consisting of different SPI-2 encoded proteins, spans the bacterial membranes and connects the pathogen to the host cytosol (modified from Grant *et al.*, 2012). Interaction of effectors with the microtubule network (black dashed lines) via host proteins like SKIP

(red connected circles) enable transmigration of enterocytes and systemic spread of *Salmonella*. The mutant (B) lacks a fully functional T3SS for SPI-2 effector protein translocation. The blocked microtubule interaction prevents transmigration, whereas intracellular replication remains possible and SCVs progressively increase in size.

5. References

Abrahams, G. L., P. Muller, and M. Hensel. 2006. 'Functional dissection of SseF, a type III effector protein involved in positioning the salmonella-containing vacuole', *Traffic*, 7: 950-65.

Achtman, M. 2008. 'Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens', *Annu Rev Microbiol*, 62: 53-70.

Achtman, M., J. Wain, F. X. Weill, S. Nair, Z. Zhou, V. Sangal, M. G. Krauland, J. L. Hale, H. Harbottle, A. Uesbeck, G. Dougan, L. H. Harrison, S. Brisse, and S. Enterica MLST Study Group. 2012. 'Multilocus sequence typing as a replacement for serotyping in Salmonella enterica', *PLoS Pathog*, 8: e1002776.

Ackermann, M., B. Stecher, N. E. Freed, P. Songhet, W. D. Hardt, and M. Doebeli. 2008. 'Self-destructive cooperation mediated by phenotypic noise', *Nature*, 454: 987-90.

Akira, S., K. Takeda, and T. Kaisho. 2001. 'Toll-like receptors: critical proteins linking innate and acquired immunity', *Nat Immunol*, 2: 675-80.

Aleksic, S., F. Heinzerling, and J. Bockemuhl. 1996. 'Human infection caused by Salmonellae of subspecies II to VI in Germany, 1977-1992', *Zentralbl Bakteriol*, 283: 391-8.

Altmeyer, M., M. Barthel, M. Eberhard, H. Rehrauer, W. D. Hardt, and M. O. Hottiger. 2010. 'Absence of poly(ADP-ribose) polymerase 1 delays the onset of Salmonella enterica serovar Typhimurium-induced gut inflammation', *Infect Immun*, 78: 3420-31.

Andrews-Polymenis, H. L., A. J. Bäumler, B. A. McCormick, and F. C. Fang. 2010. 'Taming the elephant: Salmonella biology, pathogenesis, and prevention', *Infect Immun*, 78: 2356-69.

Araujo, L. M., O. G. Ribeiro, M. Siqueira, M. De Franco, N. Starobinas, S. Massa, W. H. Cabrera, D. Mouton, M. Seman, and O. M. Ibanez. 1998. 'Innate resistance to infection by intracellular bacterial pathogens differs in mice selected for maximal or minimal acute inflammatory response', *Eur J Immunol*, 28: 2913-20.

Arevalo Sureda, E., B. Westrom, S. G. Pierzynowski, and O. Prykhodko. 2016. 'Maturation of the Intestinal Epithelial Barrier in Neonatal Rats Coincides with Decreased FcRn Expression, Replacement of Vacuolated Enterocytes and Changed Blimp-1 Expression', *PLoS One*, 11: e0164775.

Arpaia, N., J. Godec, L. Lau, K. E. Sivick, L. M. McLaughlin, M. B. Jones, T. Dracheva, S. N. Peterson, D. M. Monack, and G. M. Barton. 2011. 'TLR signaling is required for Salmonella typhimurium virulence', *Cell*, 144: 675-88.

Ayabe, T., D. P. Satchell, C. L. Wilson, W. C. Parks, M. E. Selsted, and A. J. Ouellette. 2000. 'Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria', *Nat Immunol*, 1: 113-8.

Babbar, H. S., V. M. Jaswal, R. Gupta, and A. Mahmood. 1990. 'Intestinal absorption of macromolecules and epithelial cell surface glycosylation in suckling rats nursed on mothers fed low-protein diet-I', *Biol Neonate*, 58: 104-11.

Baker, S., and G. Dougan. 2007. 'The genome of Salmonella enterica serovar Typhi', *Clin Infect Dis*, 45 Suppl 1: S29-33.

Bakowski, M. A., V. Braun, and J. H. Brumell. 2008. 'Salmonella-containing vacuoles: directing traffic and nesting to grow', *Traffic*, 9: 2022-31.

Barlow, M., and B. G. Hall. 2002. 'Origin and evolution of the AmpC beta-lactamases of Citrobacter freundii', *Antimicrob Agents Chemother*, 46: 1190-8.

Barthel, M., S. Hapfelmeier, L. Quintanilla-Martinez, M. Kremer, M. Rohde, M. Hogardt, K. Pfeffer, H. Russmann, and W. D. Hardt. 2003. 'Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host', *Infect Immun*, 71: 2839-58.

Bäumler, A., and F. C. Fang. 2013. 'Host specificity of bacterial pathogens', *Cold Spring Harbor perspectives in medicine*, 3: a010041.

Bäumler, A. J. 1997. 'The record of horizontal gene transfer in Salmonella', *Trends Microbiol*, 5: 318-22.

Bäumler, A. J., R. M. Tsolis, T. A. Ficht, and L. G. Adams. 1998. 'Evolution of host adaptation in Salmonella enterica', *Infection and immunity*, 66: 4579-87.

Bäumler, A. J., R. M. Tsolis, P. J. Valentine, T. A. Ficht, and F. Heffron. 1997. 'Synergistic effect of mutations in invA and lpfC on the ability of Salmonella typhimurium to cause murine typhoid', *Infect Immun*, 65: 2254-9.

Bellamy, R., C. Ruwende, T. Corrah, K. P. McAdam, H. C. Whittle, and A. V. Hill. 1998. 'Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans', *N Engl J Med*, 338: 640-4.

Bermudez-Brito, M., S. Munoz-Quezada, C. Gomez-Llorente, E. Matencio, M. J. Bernal, F. Romero, and A. Gil. 2012. 'Human intestinal dendritic cells decrease cytokine release against Salmonella infection in the presence of Lactobacillus paracasei upon TLR activation', *PLoS One*, 7: e43197.

Beuzon, C. R., S. Meresse, K. E. Unsworth, J. Ruiz-Albert, S. Garvis, S. R. Waterman, T. A. Ryder, E. Boucrot, and D. W. Holden. 2000. 'Salmonella maintains the integrity of its intracellular vacuole through the action of SifA', *EMBO J*, 19: 3235-49.

Beuzon, C. R., S. P. Salcedo, and D. W. Holden. 2002. 'Growth and killing of a Salmonella enterica serovar Typhimurium sifA mutant strain in the cytosol of different host cell lines', *Microbiology*, 148: 2705-15.

Biggs, H. M., R. Lester, B. Nadjm, G. Mtove, J. E. Todd, G. D. Kinabo, R. Philemon, B. Amos, A. B. Morrissey, H. Reyburn, and J. A. Crump. 2014. 'Invasive Salmonella infections in areas of high and low malaria transmission intensity in Tanzania', *Clin Infect Dis*, 58: 638-47.

Birchenough, G. M., F. Dalgakiran, L. A. Witcomb, M. E. Johansson, A. J. McCarthy, G. C. Hansson, and P. W. Taylor. 2017. 'Postnatal development of the small intestinal mucosa drives age-dependent, regio-selective susceptibility to Escherichia coli K1 infection', *Sci Rep*, 7: 83.

Birmingham, C. L., X. Jiang, M. B. Ohlson, S. I. Miller, and J. H. Brumell. 2005. 'Salmonella-induced filament formation is a dynamic phenotype induced by rapidly replicating Salmonella enterica serovar typhimurium in epithelial cells', *Infect Immun*, 73: 1204-8.

Blackwell, J. M., T. Goswami, C. A. Evans, D. Sibthorpe, N. Papo, J. K. White, S. Searle, E. N. Miller, C. S. Peacock, H. Mohammed, and M. Ibrahim. 2001. 'SLC11A1 (formerly NRAMP1) and disease resistance', *Cell Microbiol*, 3: 773-84.

Blackwell, J. M., S. Searle, T. Goswami, and E. N. Miller. 2000. 'Understanding the multiple functions of Nramp1', *Microbes Infect*, 2: 317-21.

Booth, I. R. 1985. 'Regulation of cytoplasmic pH in bacteria', *Microbiol Rev*, 49: 359-78.

Boucrot, E., C. R. Beuzon, D. W. Holden, J. P. Gorvel, and S. Meresse. 2003. 'Salmonella typhimurium SifA effector protein requires its membrane-anchoring C-terminal hexapeptide for its biological function', *J Biol Chem*, 278: 14196-202.

Bowden, S. D., G. Rowley, J. C. Hinton, and A. Thompson. 2009. 'Glucose and glycolysis are required for the successful infection of macrophages and mice by Salmonella enterica serovar typhimurium', *Infect Immun*, 77: 3117-26.

Bowe, F., C. J. Lipps, R. M. Tsolis, E. Groisman, F. Heffron, and J. G. Kusters. 1998. 'At least four percent of the Salmonella typhimurium genome is required for fatal infection of mice', *Infect Immun*, 66: 3372-7.

Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan. 2000. 'Salmonella nomenclature', *J Clin Microbiol*, 38: 2465-7.

Brown, D. E., S. J. Libby, S. M. Moreland, M. W. McCoy, T. Brabb, A. Stepanek, F. C. Fang, and C. S. Detweiler. 2013. 'Salmonella enterica causes more severe inflammatory disease in C57/BL6 Nramp1G169 mice than Sv129S6 mice', *Vet Pathol*, 50: 867-76.

Brown, N. F., B. A. Vallance, B. K. Coombes, Y. Valdez, B. A. Coburn, and B. B. Finlay. 2005. 'Salmonella pathogenicity island 2 is expressed prior to penetrating the intestine', *PLoS Pathog*, 1: e32.

Browne, S. H., P. Hasegawa, S. Okamoto, J. Fierer, and D. G. Guiney. 2008. 'Identification of Salmonella SPI-2 secretion system components required for SpvB-mediated cytotoxicity in macrophages and virulence in mice', *FEMS Immunol Med Microbiol*, 52: 194-201.

Brumell, J. H., C. M. Rosenberger, G. T. Gotto, S. L. Marcus, and B. B. Finlay. 2001. 'SifA permits survival and replication of Salmonella typhimurium in murine macrophages', *Cell Microbiol*, 3: 75-84.

Brumell, J. H., P. Tang, S. D. Mills, and B. B. Finlay. 2001. 'Characterization of Salmonella-induced filaments (Sifs) reveals a delayed interaction between Salmonella-containing vacuoles and late endocytic compartments', *Traffic*, 2: 643-53.

Brumell, J. H., P. Tang, M. L. Zaharik, and B. B. Finlay. 2002. 'Disruption of the Salmonella-containing vacuole leads to increased replication of Salmonella enterica serovar typhimurium in the cytosol of epithelial cells', *Infect Immun*, 70: 3264-70.

Bruno, V. M., S. Hannemann, M. Lara-Tejero, R. A. Flavell, S. H. Kleinstein, and J. E. Galan. 2009. 'Salmonella Typhimurium type III secretion effectors stimulate innate immune responses in cultured epithelial cells', *PLoS Pathog*, 5: e1000538.

Buckle, G. C., C. L. Walker, and R. E. Black. 2012. 'Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010', *J Glob Health*, 2: 010401.

Bumann, D., and J. Schothorst. 2017. 'Intracellular Salmonella metabolism', *Cell Microbiol*, 19.

Byrd, J. A., D. E. Corrier, J. R. Deloach, D. J. Nisbet, and L. H. Stanker. 1998. 'Horizontal Transmission of Salmonella typhimurium in Broiler Chicks', *The Journal of Applied Poultry Research*, 7: 75-80.

Canonne-Hergaux, F., J. Calafat, E. Richer, M. Cellier, S. Grinstein, N. Borregaard, and P. Gros. 2002. 'Expression and subcellular localization of NRAMP1 in human neutrophil granules', *Blood*, 100: 268-75.

Carter, P. B., and F. M. Collins. 1974. 'The route of enteric infection in normal mice', *J Exp Med*, 139: 1189-203.

Castanheira, Sónia, and Francisco García-Del Portillo. 2017. 'Salmonella Populations inside Host Cells', *Frontiers in cellular and infection microbiology*, 7: 432-32.

Chakravortty, D., I. Hansen-Wester, and M. Hensel. 2002. 'Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates', *J Exp Med*, 195: 1155-66.

Chakravortty, D., M. Rohde, L. Jager, J. Deiwick, and M. Hensel. 2005. 'Formation of a novel surface structure encoded by Salmonella Pathogenicity Island 2', *EMBO J*, 24: 2043-52.

Chassin, C., M. Kocur, J. Pott, C. U. Duerr, D. Gutle, M. Lotz, and M. W. Hornef. 2010. 'miR-146a mediates protective innate immune tolerance in the neonate intestine', *Cell Host Microbe*, 8: 358-68.

Chaudhuri, R. R., S. E. Peters, S. J. Pleasance, H. Northen, C. Willers, G. K. Paterson, D. B. Cone, A. G. Allen, P. J. Owen, G. Shalom, D. J. Stekel, I. G. Charles, and D. J. Maskell. 2009. 'Comprehensive Identification of Salmonella enterica Serovar Typhimurium Genes Required for Infection of BALB/c Mice', *PLOS Pathogens*, 5: e1000529.

Cheminay, C., D. Chakravortty, and M. Hensel. 2004. 'Role of neutrophils in murine salmonellosis', *Infect Immun*, 72: 468-77.

Cheminay, C., A. Mohlenbrink, and M. Hensel. 2005. 'Intracellular Salmonella inhibit antigen presentation by dendritic cells', *J Immunol*, 174: 2892-9.

Church, D. L., H. D. Davies, G. Cadrain, and C. L. Trevenen. 1998. 'Comparative study of three different BACTEC culture media for the detection of bacteremia in ambulatory and hospitalized children', *Can J Infect Dis*, 9: 77-82.

Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow. 1998. 'Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival', *Mol Microbiol*, 30: 175-88.

Clark, M. A., K. A. Reed, J. Lodge, J. Stephen, B. H. Hirst, and M. A. Jepson. 1996. 'Invasion of murine intestinal M cells by Salmonella typhimurium inv mutants severely deficient for invasion of cultured cells', *Infection and immunity*, 64: 4363-68.

Coburn, B., Y. Li, D. Owen, B. A. Vallance, and B. B. Finlay. 2005. 'Salmonella enterica serovar Typhimurium pathogenicity island 2 is necessary for complete virulence in a mouse model of infectious enterocolitis', *Infect Immun*, 73: 3219-27.

Coburn, B., I. Sekirov, and B. B. Finlay. 2007. 'Type III secretion systems and disease', *Clin Microbiol Rev*, 20: 535-49.

Coleman, O. I., and D. Haller. 2018. 'Bacterial Signaling at the Intestinal Epithelial Interface in Inflammation and Cancer', *Frontiers in immunology*, 8: 1927-27.

Coombes, B. K., N. F. Brown, Y. Valdez, J. H. Brumell, and B. B. Finlay. 2004. 'Expression and secretion of Salmonella pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL', *J Biol Chem*, 279: 49804-15.

Correia-Gomes, C., T. Economou, T. Bailey, P. Brazdil, L. Alban, and J. Niza-Ribeiro. 2014. 'Transmission parameters estimated for Salmonella typhimurium in swine using susceptible-infectious-resistant models and a Bayesian approach', *BMC Vet Res*, 10: 101.

Crimmins, G. T., and R. R. Isberg. 2012. 'Analyzing microbial disease at high resolution: following the fate of the bacterium during infection', *Curr Opin Microbiol*, 15: 23-7.

Crump, J. A., S. P. Luby, and E. D. Mintz. 2004. 'The global burden of typhoid fever', *Bull World Health Organ*, 82: 346-53.

Crump, J. A., and E. D. Mintz. 2010. 'Global trends in typhoid and paratyphoid Fever', *Clin Infect Dis*, 50: 241-6.

Crump, J. A., M. Sjolund-Karlsson, M. A. Gordon, and C. M. Parry. 2015. 'Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive Salmonella Infections', *Clin Microbiol Rev*, 28: 901-37.

Cuellar-Mata, P., N. Jabado, J. Liu, W. Furuya, B. B. Finlay, P. Gros, and S. Grinstein. 2002. 'Nramp1 modifies the fusion of Salmonella typhimurium-containing vacuoles with cellular endomembranes in macrophages', *J Biol Chem*, 277: 2258-65.

D'Costa, V. M., V. Braun, M. Landekic, R. Shi, A. Proteau, L. McDonald, M. Cygler, S. Grinstein, and J. H. Brumell. 2015. 'Salmonella Disrupts Host Endocytic Trafficking by SopD2-Mediated Inhibition of Rab7', *Cell Rep*, 12: 1508-18.

Dalebroux, Z. D., and S. I. Miller. 2014. 'Salmonellae PhoPQ regulation of the outer membrane to resist innate immunity', *Curr Opin Microbiol*, 17: 106-13.

Dandekar, T., F. Astrid, P. Jasmin, and M. Hensel. 2012. 'Salmonella enterica: a surprisingly well-adapted intracellular lifestyle', *Front Microbiol*, 3: 164.

Dandekar, T., A. Fieselmann, E. Fischer, J. Popp, M. Hensel, and J. Noster. 2014. 'Salmonella-how a metabolic generalist adopts an intracellular lifestyle during infection', *Front Cell Infect Microbiol*, 4: 191.

Datsenko, K. A., and B. L. Wanner. 2000. 'One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products', *Proc Natl Acad Sci U S A*, 97: 6640-5.

Davies, R. H., and C. Wray. 1996. 'Persistence of Salmonella enteritidis in poultry units and poultry food', *Br Poult Sci*, 37: 589-96.

de Santa Barbara, P., G. R. van den Brink, and D. J. Roberts. 2003. 'Development and differentiation of the intestinal epithelium', *Cell Mol Life Sci*, 60: 1322-32.

Deiwick, J., and M. Hensel. 1999. 'Regulation of virulence genes by environmental signals in Salmonella typhimurium', *Electrophoresis*, 20: 813-7.

Deiwick, J., T. Nikolaus, S. Erdogan, and M. Hensel. 1999. 'Environmental regulation of Salmonella pathogenicity island 2 gene expression', *Mol Microbiol*, 31: 1759-73.

Deiwick, J., T. Nikolaus, J. E. Shea, C. Gleeson, D. W. Holden, and M. Hensel. 1998. 'Mutations in Salmonella pathogenicity island 2 (SPI2) genes affecting transcription of SPI1 genes and resistance to antimicrobial agents', *Journal of bacteriology*, 180: 4775-80.

Deiwick, J., S. P. Salcedo, E. Boucrot, S. M. Gilliland, T. Henry, N. Petermann, S. R. Waterman, J. P. Gorvel, D. W. Holden, and S. Meresse. 2006. 'The translocated Salmonella effector proteins SseF and SseG interact and are required to establish an intracellular replication niche', *Infect Immun*, 74: 6965-72.

Dekaney, C. M., F. W. Bazer, and L. A. Jaeger. 1997. 'Mucosal morphogenesis and cytodifferentiation in fetal porcine small intestine', *Anat Rec*, 249: 517-23.

den Bakker, H. C., A. I. Moreno Switt, G. Govoni, C. A. Cummings, M. L. Ranieri, L. Degoricija, K. Hoelzer, L. D. Rodriguez-Rivera, S. Brown, E. Bolchacova, M. R. Furtado, and M. Wiedmann. 2011.

'Genome sequencing reveals diversification of virulence factor content and possible host adaptation in distinct subpopulations of Salmonella enterica', *BMC Genomics*, 12: 425.

Desai, P. T., S. Porwollik, F. Long, P. Cheng, A. Wollam, V. Bhonagiri-Palsikar, K. Hallsworth-Pepin, S. W. Clifton, G. M. Weinstock, and M. McClelland. 2013. 'Evolutionary Genomics of Salmonella enterica Subspecies', *MBio*, 4.

Diacovich, L., A. Dumont, D. Lafitte, E. Soprano, A. A. Guilhon, C. Bignon, J. P. Gorvel, Y. Bourne, and S. Meresse. 2009. 'Interaction between the SifA virulence factor and its host target SKIP is essential for Salmonella pathogenesis', *J Biol Chem*, 284: 33151-60.

Diard, M., V. Garcia, L. Maier, M. N. Remus-Emsermann, R. R. Regoes, M. Ackermann, and W. D. Hardt. 2013. 'Stabilization of cooperative virulence by the expression of an avirulent phenotype', *Nature*, 494: 353-6.

Domingues, L., D. W. Holden, and L. J. Mota. 2014. 'The Salmonella effector SteA contributes to the control of membrane dynamics of Salmonella-containing vacuoles', *Infect Immun*, 82: 2923-34.

Dos Santos, A. M. P., R. G. Ferrari, and C. A. Conte-Junior. 2019. 'Virulence Factors in Salmonella Typhimurium: The Sagacity of a Bacterium', *Curr Microbiol*, 76: 762-73.

Drevets, D. A., P. J. Leenen, and R. A. Greenfield. 2004. 'Invasion of the central nervous system by intracellular bacteria', *Clin Microbiol Rev*, 17: 323-47.

Dumont, A., E. Boucrot, S. Drevensek, V. Daire, J. P. Gorvel, C. Pous, D. W. Holden, and S. Meresse. 2010. 'SKIP, the host target of the Salmonella virulence factor SifA, promotes kinesin-1-dependent vacuolar membrane exchanges', *Traffic*, 11: 899-911.

Dupont, A., Y. Kaconis, I. Yang, T. Albers, S. Woltemate, L. Heinbockel, M. Andersson, S. Suerbaum, K. Brandenburg, and M. W. Hornef. 2015. 'Intestinal mucus affinity and biological activity of an orally administered antibacterial and anti-inflammatory peptide', *Gut*, 64: 222-32.

Eastwood, G. L. 1977. 'Gastrointestinal epithelial renewal', *Gastroenterology*, 72: 962-75.

Eberth, C. J. %J Archiv für pathologische Anatomie und Physiologie und für klinische Medicin. 1880. 'Die Organismen in den Organen bei Typhus abdominalis', 81: 58-74.

Edwards, P. R., D. W. Bruner, and A. B. Moran. 1948. 'Further studies on the occurrence and distribution of Salmonella types in the United States', *J Infect Dis*, 83: 220-31.

Eguale, T. 2018. 'Non-typhoidal Salmonella serovars in poultry farms in central Ethiopia: prevalence and antimicrobial resistance', *BMC Vet Res*, 14: 217.

Ehrbar, K., and W. D. Hardt. 2005. 'Bacteriophage-encoded type III effectors in Salmonella enterica subspecies 1 serovar Typhimurium', *Infect Genet Evol*, 5: 1-9.

Eng, SK., P. Pusparajah, NS. Ab Mutalib, H L. Ser, KG. Chan, and L H. Lee. 2015. 'Salmonella : A review on pathogenesis, epidemiology and antibiotic resistance', *Frontiers in Life Science*, 8: 1-10.

Faber, F., P. Thiennimitr, L. Spiga, M. X. Byndloss, Y. Litvak, S. Lawhon, H. L. Andrews-Polymenis, S. E. Winter, and A. J. Baumler. 2017. 'Respiration of Microbiota-Derived 1,2-propanediol Drives Salmonella Expansion during Colitis', *PLoS Pathog*, 13: e1006129.

Farooqui, A., A. Khan, and S. U. Kazmi. 2009. 'Investigation of a community outbreak of typhoid fever associated with drinking water', *BMC Public Health*, 9: 476.

Fass, E., and E. A. Groisman. 2009. 'Control of Salmonella pathogenicity island-2 gene expression', *Curr Opin Microbiol*, 12: 199-204.

Feasey, N. A., D. Everett, E. B. Faragher, A. Roca-Feltrer, A. Kang'ombe, B. Denis, M. Kerac, E. Molyneux, M. Molyneux, A. Jahn, M. A. Gordon, and R. S. Heyderman. 2015. 'Modelling the Contributions of Malaria, HIV, Malnutrition and Rainfall to the Decline in Paediatric Invasive Non-typhoidal Salmonella Disease in Malawi', *PLoS neglected tropical diseases*, 9: e0003979.

Fedorka-Cray, P. J., S. C. Whipp, R. E. Isaacson, N. Nord, and K. Lager. 1994. 'Transmission of Salmonella typhimurium to swine', *Vet Microbiol*, 41: 333-44.

Feuillet, V., S. Medjane, I. Mondor, O. Demaria, P. P. Pagni, J. E. Galan, R. A. Flavell, and L. Alexopoulou. 2006. 'Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria', *Proc Natl Acad Sci U S A*, 103: 12487-92.

Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron. 1986. 'Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent', *Proceedings of the National Academy of Sciences of the United States of America*, 83: 5189-93.

Figueira, R., and D. W. Holden. 2012. 'Functions of the Salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors', *Microbiology*, 158: 1147-61.

Finlay, B. B., and J. H. Brumell. 2000. 'Salmonella interactions with host cells: in vitro to in vivo', *Philos Trans R Soc Lond B Biol Sci*, 355: 623-31.

Finn, S., O. Condell, P. McClure, A. Amézquita, and S. Fanning. 2013. 'Mechanisms of survival, responses and sources of Salmonella in low-moisture environments', *Front Microbiol*, 4: 331.

Fitzsimmons, L. F., L. Liu, J. S. Kim, J. Jones-Carson, and A. Vazquez-Torres. 2018. 'Salmonella Reprograms Nucleotide Metabolism in Its Adaptation to Nitrosative Stress', *MBio*, 9.

Fluit, A. C. 2005. 'Towards more virulent and antibiotic-resistant Salmonella?', *FEMS Immunol Med Microbiol*, 43: 1-11.

Foley, J. E., U. Orgad, D. C. Hirsh, A. Poland, and N. C. Pedersen. 1999. 'Outbreak of fatal salmonellosis in cats following use of a high-titer modified-live panleukopenia virus vaccine', *J Am Vet Med Assoc*, 214: 67-70, 43-4.

Fookes, M., G. N. Schroeder, G. C. Langridge, C. J. Blondel, C. Mammina, T. R. Connor, H. Seth-Smith, G. S. Vernikos, K. S. Robinson, M. Sanders, N. K. Petty, R. A. Kingsley, A. J. Baumler, S. P. Nuccio, I. Contreras, C. A. Santiviago, D. Maskell, P. Barrow, T. Humphrey, A. Nastasi, M. Roberts, G. Frankel, J. Parkhill, G. Dougan, and N. R. Thomson. 2011. 'Salmonella bongori provides insights into the evolution of the Salmonellae', *PLoS Pathog*, 7: e1002191.

Forbes, J. R., and P. Gros. 2001. 'Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions', *Trends Microbiol*, 9: 397-403.

———. 2003. 'Iron, manganese, and cobalt transport by Nramp1 (Slc11a1) and Nramp2 (Slc11a2) expressed at the plasma membrane', *Blood*, 102: 1884-92.

Forest, C. G., E. Ferraro, S. C. Sabbagh, and F. Daigle. 2010. 'Intracellular survival of Salmonella enterica serovar Typhi in human macrophages is independent of Salmonella pathogenicity island (SPI)-2', *Microbiology*, 156: 3689-98.

Foster, J. W., and H. K. Hall. 1990. 'Adaptive acidification tolerance response of Salmonella typhimurium', *J Bacteriol*, 172: 771-8.

———. 1991. 'Inducible pH homeostasis and the acid tolerance response of Salmonella typhimurium', *J Bacteriol*, 173: 5129-35.

Fremont-Rahl, J. J., Z. Ge, C. Umana, M. T. Whary, N. S. Taylor, S. Muthupalani, M. C. Carey, J. G. Fox, and K. J. Maurer. 2013. 'An Analysis of the Role of the Indigenous Microbiota in Cholesterol Gallstone Pathogenesis', *PLoS One*, 8: e70657.

Fritsche, G., M. Nairz, S. J. Libby, F. C. Fang, and G. Weiss. 2012. 'Slc11a1 (Nramp1) impairs growth of Salmonella enterica serovar typhimurium in macrophages via stimulation of lipocalin-2 expression', *J Leukoc Biol*, 92: 353-9.

Fulde, M., F. Sommer, B. Chassaing, K. van Vorst, A. Dupont, M. Hensel, M. Basic, R. Klopfleisch, P. Rosenstiel, A. Bleich, F. Backhed, A. T. Gewirtz, and M. W. Hornef. 2018. 'Neonatal selection by Toll-like receptor 5 influences long-term gut microbiota composition', *Nature*, 560: 489-93.

Gal-Mor, O. 2019. 'Persistent Infection and Long-Term Carriage of Typhoidal and Nontyphoidal Salmonellae', *Clin Microbiol Rev*, 32.

Gal-Mor, O., E. C. Boyle, and G. A. Grassl. 2014. 'Same species, different diseases: how and why typhoidal and non-typhoidal Salmonella enterica serovars differ', *Front Microbiol*, 5: 391.

Gal-Mor, O., and B. B. Finlay. 2006. 'Pathogenicity islands: a molecular toolbox for bacterial virulence', *Cell Microbiol*, 8: 1707-19.

Galan, J. E. 2001. 'Salmonella interactions with host cells: type III secretion at work', *Annu Rev Cell Dev Biol*, 17: 53-86.

Galan, J. E., and R. Curtiss, 3rd. 1989. 'Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells', *Proc Natl Acad Sci U S A*, 86: 6383-7.

Galanis, E., D. M. Lo Fo Wong, M. E. Patrick, N. Binsztein, A. Cieslik, T. Chalermchikit, A. Aidara-Kane, A. Ellis, F. J. Angulo, and H. C. Wegener. 2006. 'Web-based surveillance and global Salmonella distribution, 2000-2002', *Emerg Infect Dis*, 12: 381-8.

Galyov, E. E., M. W. Wood, R. Rosqvist, P. B. Mullan, P. R. Watson, S. Hedges, and T. S. Wallis. 1997. 'A secreted effector protein of Salmonella dublin is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa', *Mol Microbiol*, 25: 903-12.

Garai, P., S. A. Marathe, and D. Chakravortty. 2011. 'Effectors of Salmonella pathogenicity island 2: An island crucial to the life of Salmonella', *Virulence*, 2: 177-80.

Garcia-del Portillo, F., and B. B. Finlay. 1995. 'Targeting of Salmonella typhimurium to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors', *J Cell Biol*, 129: 81-97.

Garcia-del Portillo, F., M. B. Zwick, K. Y. Leung, and B. B. Finlay. 1993. 'Salmonella induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells', *Proc Natl Acad Sci U S A*, 90: 10544-8.

Garmendia, J., C. R. Beuzon, J. Ruiz-Albert, and D. W. Holden. 2003. 'The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the Salmonella typhimurium SPI-2 type III secretion system', *Microbiology*, 149: 2385-96.

Geddes, K., M. Worley, G. Niemann, and F. Heffron. 2005. 'Identification of new secreted effectors in Salmonella enterica serovar Typhimurium', *Infect Immun*, 73: 6260-71.

Gerlach, R. G., and M. Hensel. 2007. 'Salmonella pathogenicity islands in host specificity, host pathogen-interactions and antibiotics resistance of Salmonella enterica', *Berl Munch Tierarztl Wochenschr*, 120: 317-27.

Gibani, M. M., C. Britto, and A. J. Pollard. 2018. 'Typhoid and paratyphoid fever: a call to action', *Curr Opin Infect Dis*, 31: 440-48.

Gibani, M. M., E. Jones, A. Barton, C. Jin, J. Meek, S. Camara, U. Galal, E. Heinz, Y. Rosenberg-Hasson, G. Obermoser, C. Jones, D. Campbell, C. Black, H. Thomaides-Brears, C. Darlow, C. Dold, L. Silva-Reyes, L. Blackwell, M. Lara-Tejero, X. Jiao, G. Stack, C. J. Blohmke, J. Hill, B. Angus, G. Dougan, J. Galan, and A. J. Pollard. 2019. 'Investigation of the role of typhoid toxin in acute typhoid fever in a human challenge model', *Nat Med*, 25: 1082-88.

Gomez, T. M., Y. Motarjemi, S. Miyagawa, F. K. Kaferstein, and K. Stohr. 1997. 'Foodborne salmonellosis', *World Health Stat Q*, 50: 81-9.

Gordon, J. I., and M. L. Hermiston. 1994. 'Differentiation and self-renewal in the mouse gastrointestinal epithelium', *Curr Opin Cell Biol*, 6: 795-803.

Gordon, Melita A. 2011. 'Invasive nontyphoidal Salmonella disease: epidemiology, pathogenesis and diagnosis', *Current opinion in infectious diseases*, 24: 484-89.

Govoni, G., S. Vidal, S. Gauthier, E. Skamene, D. Malo, and P. Gros. 1996. 'The Bcg/Ity/Lsh locus: genetic transfer of resistance to infections in C57BL/6J mice transgenic for the Nramp1 Gly169 allele', *Infect Immun*, 64: 2923-9.

Graham, S. M., A. L. Walsh, E. M. Molyneux, A. J. Phiri, and M. E. Molyneux. 2000. 'Clinical presentation of non-typhoidal Salmonella bacteraemia in Malawian children', *Trans R Soc Trop Med Hyg*, 94: 310-4.

Grant, A. J., F. J. Morgan, T. J. McKinley, G. L. Foster, D. J. Maskell, and P. Mastroeni. 2012. 'Attenuated Salmonella Typhimurium lacking the pathogenicity island-2 type 3 secretion system grow to high bacterial numbers inside phagocytes in mice', *PLoS Pathog*, 8: e1003070.

Grant, A. J., O. Restif, T. J. McKinley, M. Sheppard, D. J. Maskell, and P. Mastroeni. 2008. 'Modelling within-host spatiotemporal dynamics of invasive bacterial disease', *PLoS Biol*, 6: e74.

Groisman, E. A., and H. Ochman. 1996. 'Pathogenicity islands: bacterial evolution in quantum leaps', *Cell*, 87: 791-4.

———. 1997. 'How Salmonella became a pathogen', *Trends Microbiol*, 5: 343-9.

Gruenheid, S., E. Pinner, M. Desjardins, and P. Gros. 1997. 'Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome', *The Journal of experimental medicine*, 185: 717-30.

Guibourdenche, M., P. Roggentin, M. Mikoleit, P. I. Fields, J. Bockemuhl, P. A. Grimont, and F. X. Weill. 2010. 'Supplement 2003-2007 (No. 47) to the White-Kauffmann-Le Minor scheme', *Res Microbiol*, 161: 26-9.

Guignot, J., E. Caron, C. Beuzon, C. Bucci, J. Kagan, C. Roy, and D. W. Holden. 2004. 'Microtubule motors control membrane dynamics of Salmonella-containing vacuoles', *J Cell Sci*, 117: 1033-45.

Guiney, D. G., and J. Fierer. 2011. 'The Role of the spv Genes in Salmonella Pathogenesis', *Front Microbiol*, 2: 129.

Gulig, P. A., H. Danbara, D. G. Guiney, A. J. Lax, F. Norel, and M. Rhen. 1993. 'Molecular analysis of spv virulence genes of the Salmonella virulence plasmids', *Mol Microbiol*, 7: 825-30.

Gulig, P. A., and T. J. Doyle. 1993. 'The Salmonella typhimurium virulence plasmid increases the growth rate of salmonellae in mice', *Infect Immun*, 61: 504-11.

Habte, L., E. Tadesse, G. Ferede, and A. Amsalu. 2018. 'Typhoid fever: clinical presentation and associated factors in febrile patients visiting Shashemene Referral Hospital, southern Ethiopia', *BMC Res Notes*, 11: 605.

Hackam, D. J., O. D. Rotstein, W. Zhang, S. Gruenheid, P. Gros, and S. Grinstein. 1998. 'Host resistance to intracellular infection: mutation of natural resistance-associated macrophage protein 1 (Nramp1) impairs phagosomal acidification', *J Exp Med*, 188: 351-64.

Hale, C. R., E. Scallan, A. B. Cronquist, J. Dunn, K. Smith, T. Robinson, S. Lathrop, M. Tobin-D'Angelo, and P. Clogher. 2012. 'Estimates of enteric illness attributable to contact with animals and their environments in the United States', *Clin Infect Dis*, 54 Suppl 5: S472-9.

Hansen-Wester, I., B. Stecher, and M. Hensel. 2002. 'Type III secretion of Salmonella enterica serovar Typhimurium translocated effectors and SseFG', *Infect Immun*, 70: 1403-9.

Hapfelmeier, S., B. Stecher, M. Barthel, M. Kremer, A. J. Muller, M. Heikenwalder, T. Stallmach, M. Hensel, K. Pfeffer, S. Akira, and W. D. Hardt. 2005. 'The Salmonella pathogenicity island (SPI)-2 and SPI-1 type III secretion systems allow Salmonella serovar typhimurium to trigger colitis via MyD88-dependent and MyD88-independent mechanisms', *J Immunol*, 174: 1675-85.

Haraga, A., and S. I. Miller. 2003. 'A Salmonella enterica serovar typhimurium translocated leucine-rich repeat effector protein inhibits NF-kappa B-dependent gene expression', *Infect Immun*, 71: 4052-8.

———. 2006. 'A Salmonella type III secretion effector interacts with the mammalian serine/threonine protein kinase PKN1', *Cell Microbiol*, 8: 837-46.

Haraga, A., M. B. Ohlson, and S. I. Miller. 2008. 'Salmonellae interplay with host cells', *Nat Rev Microbiol*, 6: 53-66.

Hardy, A. 1999. 'Food, hygiene, and the laboratory. A short history of food poisoning in Britain, circa 1850-1950', *Soc Hist Med*, 12: 293-311.

Harper, J., A. Mould, R. M. Andrews, E. K. Bikoff, and E. J. Robertson. 2011. 'The transcriptional repressor Blimp1/Prdm1 regulates postnatal reprogramming of intestinal enterocytes', *Proc Natl Acad Sci U S A*, 108: 10585-90.

Harrison, R. E., J. H. Brumell, A. Khandani, C. Bucci, C. C. Scott, X. Jiang, B. B. Finlay, and S. Grinstein. 2004. 'Salmonella impairs RILP recruitment to Rab7 during maturation of invasion vacuoles', *Mol Biol Cell*, 15: 3146-54.

Harrison, R. E., C. Bucci, O. V. Vieira, T. A. Schroer, and S. Grinstein. 2003. 'Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP', *Mol Cell Biol*, 23: 6494-506.

Hartmann, F. A., R. J. Callan, S. M. McGuirk, and S. E. West. 1996. 'Control of an outbreak of salmonellosis caused by drug-resistant Salmonella anatum in horses at a veterinary hospital and measures to prevent future infections', *J Am Vet Med Assoc*, 209: 629-31.

Hase, K., K. Kawano, T. Nochi, G. S. Pontes, S. Fukuda, M. Ebisawa, K. Kadokura, T. Tobe, Y. Fujimura, S. Kawano, A. Yabashi, S. Waguri, G. Nakato, S. Kimura, T. Murakami, M. Iimura, K. Hamura, S.

Fukuoka, A. W. Lowe, K. Itoh, H. Kiyono, and H. Ohno. 2009. 'Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response', *Nature*, 462: 226-30.

Havelaar, A. H., M. D. Kirk, P. R. Torgerson, H. J. Gibb, T. Hald, R. J. Lake, N. Praet, D. C. Bellinger, N. R. de Silva, N. Gargouri, N. Speybroeck, A. Cawthorne, C. Mathers, C. Stein, F. J. Angulo, B. Devleesschauwer, and Group World Health Organization Foodborne Disease Burden Epidemiology Reference. 2015. 'World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010', *PLoS Med*, 12: e1001923.

Heithoff, D. M., C. P. Conner, U. Hentschel, F. Govantes, P. C. Hanna, and M. J. Mahan. 1999. 'Coordinate intracellular expression of Salmonella genes induced during infection', *Journal of bacteriology*, 181: 799-807.

Helaine, S., J. A. Thompson, K. G. Watson, M. Liu, C. Boyle, and D. W. Holden. 2010. 'Dynamics of intracellular bacterial replication at the single cell level', *Proc Natl Acad Sci U S A*, 107: 3746-51.

Hendriksen, S. W., K. Orsel, J. A. Wagenaar, A. Miko, and E. van Duijkeren. 2004. 'Animal-to-human transmission of Salmonella Typhimurium DT104A variant', *Emerg Infect Dis*, 10: 2225-7.

Henry, T., C. Couillault, P. Rockenfeller, E. Boucrot, A. Dumont, N. Schroeder, A. Hermant, L. A. Knodler, P. Lecine, O. Steele-Mortimer, J. P. Borg, J. P. Gorvel, and S. Meresse. 2006. 'The Salmonella effector protein PipB2 is a linker for kinesin-1', *Proc Natl Acad Sci U S A*, 103: 13497-502.

Hensel, M., T. Nikolaus, and C. Egelseer. 1999. 'Molecular and functional analysis indicates a mosaic structure of Salmonella pathogenicity island 2', *Mol Microbiol*, 31: 489-98.

Hensel, M., J. E. Shea, A. J. Baumler, C. Gleeson, F. Blattner, and D. W. Holden. 1997. 'Analysis of the boundaries of Salmonella pathogenicity island 2 and the corresponding chromosomal region of Escherichia coli K-12', *J Bacteriol*, 179: 1105-11.

Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden. 1995. 'Simultaneous identification of bacterial virulence genes by negative selection', *Science*, 269: 400-3.

Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden. 1998. 'Genes encoding putative effector proteins of the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages', *Mol Microbiol*, 30: 163-74.

Hoelzer, K., A. I. Moreno Switt, and M. Wiedmann. 2011. 'Animal contact as a source of human non-typhoidal salmonellosis', *Vet Res*, 42: 34.

Holt, K. E., J. Parkhill, C. J. Mazzoni, P. Roumagnac, F. X. Weill, I. Goodhead, R. Rance, S. Baker, D. J. Maskell, J. Wain, C. Dolecek, M. Achtman, and G. Dougan. 2008. 'High-throughput sequencing provides insights into genome variation and evolution in Salmonella Typhi', *Nat Genet*, 40: 987-93.

Hornef, M. W., and M. Fulde. 2014. 'Ontogeny of intestinal epithelial innate immune responses', *Front Immunol*, 5: 474.

Hornick, R. B., S. E. Greisman, T. E. Woodward, H. L. DuPont, A. T. Dawkins, and M. J. Snyder. 1970. 'Typhoid fever: pathogenesis and immunologic control', *N Engl J Med*, 283: 686-91.

Houghteling, P. D., and W. A. Walker. 2015. 'Why is initial bacterial colonization of the intestine important to infants' and children's health?', *J Pediatr Gastroenterol Nutr*, 60: 294-307.

Hughes, L. E., E. A. Gibson, H. E. Roberts, E. T. Davies, G. Davies, and W. J. Sojka. 1971. 'Bovine salmonellosis in England and Wales', *Br Vet J*, 127: 225-38.

Humphrey, T. J., G. C. Mead, and B. Rowe. 1988. 'Poultry meat as a source of human salmonellosis in England and Wales. Epidemiological overview', *Epidemiol Infect*, 100: 175-84.

Ibarra, J. A., and O. Steele-Mortimer. 2009. 'Salmonella--the ultimate insider. Salmonella virulence factors that modulate intracellular survival', *Cell Microbiol*, 11: 1579-86.

Ilg, K., K. Endt, B. Misselwitz, B. Stecher, M. Aebi, and W. D. Hardt. 2009. 'O-antigen-negative Salmonella enterica serovar Typhimurium is attenuated in intestinal colonization but elicits colitis in streptomycin-treated mice', *Infect Immun*, 77: 2568-75.

Ilyas, B., C. N. Tsai, and B. K. Coombes. 2017. 'Evolution of Salmonella-Host Cell Interactions through a Dynamic Bacterial Genome', *Front Cell Infect Microbiol*, 7: 428.

Issenhuth-Jeanjean, S., P. Roggentin, M. Mikoleit, M. Guibourdenche, E. de Pinna, S. Nair, P. I. Fields, and F. X. Weill. 2014. 'Supplement 2008-2010 (no. 48) to the White-Kauffmann-Le Minor scheme', *Res Microbiol*, 165: 526-30.

Iwasaki, A., and R. Medzhitov. 2004. 'Toll-like receptor control of the adaptive immune responses', *Nat Immunol*, 5: 987-95.

Jackson, L. K., P. Nawabi, C. Hentea, E. A. Roark, and K. Haldar. 2008. 'The Salmonella virulence protein SifA is a G protein antagonist', *Proc Natl Acad Sci U S A*, 105: 14141-6.

Jajere, S. M. 2019. 'A review of Salmonella enterica with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance', *Vet World*, 12: 504-21.

Janeway, C. A., Jr. 1989. 'Approaching the asymptote? Evolution and revolution in immunology', *Cold Spring Harb Symp Quant Biol*, 54 Pt 1: 1-13.

Jee, J., R. Mourya, P. Shivakumar, L. Fei, M. Wagner, and J. A. Bezerra. 2017. 'Cxcr2 signaling and the microbiome suppress inflammation, bile duct injury, and the phenotype of experimental biliary atresia', *PLoS One*, 12: e0182089.

Jepson, M. A., and M. A. Clark. 2001. 'The role of M cells in Salmonella infection', *Microbes Infect*, 3: 1183-90.

Johnson, R., E. Mylona, and G. Frankel. 2018. 'Typhoidal Salmonella: Distinctive virulence factors and pathogenesis', *Cell Microbiol*, 20: e12939.

Jones, B. D., N. Ghori, and S. Falkow. 1994. 'Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches', *J Exp Med*, 180: 15-23.

Juven, B. J., N. A. Cox, J. S. Bailey, J. E. Thomson, O. W. Charles, and J. V. Shutze. 1984. 'Survival of Salmonella in Dry Food and Feed', *J Food Prot*, 47: 445-48.

Kaiser, P., M. Diard, B. Stecher, and W. D. Hardt. 2012. 'The streptomycin mouse model for Salmonella diarrhea: functional analysis of the microbiota, the pathogen's virulence factors, and the host's mucosal immune response', *Immunol Rev*, 245: 56-83.

Kaiser, P., R. R. Regoes, T. Dolowschiak, S. Y. Wotzka, J. Lengefeld, E. Slack, A. J. Grant, M. Ackermann, and W. D. Hardt. 2014. 'Cecum lymph node dendritic cells harbor slow-growing bacteria phenotypically tolerant to antibiotic treatment', *PLoS Biol*, 12: e1001793.

Kaiser, P., E. Slack, A. J. Grant, W. D. Hardt, and R. R. Regoes. 2013. 'Lymph node colonization dynamics after oral Salmonella Typhimurium infection in mice', *PLoS Pathog*, 9: e1003532.

Kawai, T., and S. Akira. 2005. 'Pathogen recognition with Toll-like receptors', *Curr Opin Immunol*, 17: 338-44.

Keerthirathne, T. P., K. Ross, H. Fallowfield, and H. Whiley. 2016. 'A Review of Temperature, pH, and Other Factors that Influence the Survival of Salmonella in Mayonnaise and Other Raw Egg Products', *Pathogens*, 5.

Kelly, D., R. Begbie, and T. P. King. 1992. 'Postnatal intestinal development', *BSAP Occasional Publication*, 15: 63-79.

Kim, Y. M., B. J. Schmidt, A. S. Kidwai, M. B. Jones, B. L. Deatherage Kaiser, H. M. Brewer, H. D. Mitchell, B. O. Palsson, J. E. McDermott, F. Heffron, R. D. Smith, S. N. Peterson, C. Ansong, D. R. Hyduke, T. O. Metz, and J. N. Adkins. 2013. 'Salmonella modulates metabolism during growth under conditions that induce expression of virulence genes', *Mol Biosyst*, 9: 1522-34.

Kingsley, R. A., and A. J. Bäumler. 2000. 'Host adaptation and the emergence of infectious disease: the Salmonella paradigm', *Mol Microbiol*, 36: 1006-14.

Klotz, S. A., J. H. Jorgensen, F. J. Buckwold, and P. C. Craven. 1984. 'Typhoid fever. An epidemic with remarkably few clinical signs and symptoms', *Arch Intern Med*, 144: 533-7.

Knodler, L. A. 2015. 'Salmonella enterica: living a double life in epithelial cells', *Curr Opin Microbiol*, 23: 23-31.

Knodler, L. A., and O. Steele-Mortimer. 2005. 'The Salmonella effector PipB2 affects late endosome/lysosome distribution to mediate Sif extension', *Mol Biol Cell*, 16: 4108-23.

Knodler, L. A., B. A. Vallance, J. Celli, S. Winfree, B. Hansen, M. Montero, and O. Steele-Mortimer. 2010. 'Dissemination of invasive Salmonella via bacterial-induced extrusion of mucosal epithelia', *Proc Natl Acad Sci U S A*, 107: 17733-8.

Knodler, L. A., B. A. Vallance, M. Hensel, D. Jackel, B. B. Finlay, and O. Steele-Mortimer. 2003. 'Salmonella type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes', *Mol Microbiol*, 49: 685-704.

Kolodziejek, A. M., and S. I. Miller. 2015. 'Salmonella modulation of the phagosome membrane, role of SseJ', *Cell Microbiol*, 17: 333-41.

Kraehenbuhl, J. P., C. Bron, and B. Sordat. 1979. 'Transfer of humoral secretory and cellular immunity from mother to offspring', *Curr Top Pathol*, 66: 105-57.

Krieger, V., D. Liebl, Y. Zhang, R. Rajashekar, P. Chlanda, K. Giesker, D. Chikkaballi, and M. Hensel. 2014. 'Reorganization of the endosomal system in Salmonella-infected cells: the ultrastructure of Salmonella-induced tubular compartments', *PLoS Pathog*, 10: e1004374.

Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S. I. Aizawa. 1998. 'Supramolecular structure of the Salmonella typhimurium type III protein secretion system', *Science*, 280: 602-5.

Kuhle, V., and M. Hensel. 2002. 'SseF and SseG are translocated effectors of the type III secretion system of Salmonella pathogenicity island 2 that modulate aggregation of endosomal compartments', *Cell Microbiol*, 4: 813-24.

———. 2004. 'Cellular microbiology of intracellular Salmonella enterica: functions of the type III secretion system encoded by Salmonella pathogenicity island 2', *Cell Mol Life Sci*, 61: 2812-26.

Kuhle, V., D. Jackel, and M. Hensel. 2004. 'Effector proteins encoded by Salmonella pathogenicity island 2 interfere with the microtubule cytoskeleton after translocation into host cells', *Traffic*, 5: 356-70.

Kumagai, N., R. Baba, Y. Sakuma, K. Arita, M. Shinohara, M. Kourogi, S. Fujimoto, and M. Fujita. 2011. 'Origin of the apical transcytic membrane system in jejunal absorptive cells of neonates', *Med Mol Morphol*, 44: 71-8.

Langridge, G. C., M. Fookes, T. R. Connor, T. Feltwell, N. Feasey, B. N. Parsons, H. M. Seth-Smith, L. Barquist, A. Stedman, T. Humphrey, P. Wigley, S. E. Peters, D. J. Maskell, J. Corander, J. A. Chabalgoity, P. Barrow, J. Parkhill, G. Dougan, and N. R. Thomson. 2015. 'Patterns of genome evolution that have accompanied host adaptation in Salmonella', *Proc Natl Acad Sci U S A*, 112: 863-8.

Langridge, G. C., J. Wain, and S. Nair. 2012. 'Invasive Salmonellosis in Humans', *EcoSal Plus*, 5.

LaRock, D. L., A. Chaudhary, and S. I. Miller. 2015. 'Salmonellae interactions with host processes', *Nat Rev Microbiol*, 13: 191-205.

Lawley, T. D., D. M. Bouley, Y. E. Hoy, C. Gerke, D. A. Relman, and D. M. Monack. 2008. 'Host transmission of Salmonella enterica serovar Typhimurium is controlled by virulence factors and indigenous intestinal microbiota', *Infect Immun*, 76: 403-16.

Lee, C. A., M. Silva, A. M. Siber, A. J. Kelly, E. Galyov, and B. A. McCormick. 2000. 'A secreted Salmonella protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration', *Proc Natl Acad Sci U S A*, 97: 12283-8.

Lei, L., W. Wang, C. Xia, and F. Liu. 2016. 'Salmonella Virulence Factor SsrAB Regulated Factor Modulates Inflammatory Responses by Enhancing the Activation of NF-kappaB Signaling Pathway', *J Immunol*, 196: 792-802.

Lestari, S. I., F. Han, F. Wang, and B. Ge. 2009. 'Prevalence and antimicrobial resistance of Salmonella serovars in conventional and organic chickens from Louisiana retail stores', *J Food Prot*, 72: 1165-72.

Leung, K Y, and B B Finlay. 1991. 'Intracellular replication is essential for the virulence of Salmonella typhimurium', *Proceedings of the National Academy of Sciences*, 88: 11470-74.

Lim, C. H., S. Voedisch, B. Wahl, S. F. Rouf, R. Geffers, M. Rhen, and O. Pabst. 2014. 'Independent bottlenecks characterize colonization of systemic compartments and gut lymphoid tissue by salmonella', *PLoS Pathog*, 10: e1004270.

Lim, S., M. Kim, J. Choi, and S. Ryu. 2010. 'A mutation in tdcA attenuates the virulence of Salmonella enterica serovar Typhimurium', *Mol Cells*, 29: 509-17.

Liss, V., A. L. Swart, A. Kehl, N. Hermanns, Y. Zhang, D. Chikkaballi, N. Bohles, J. Deiwick, and M. Hensel. 2017. 'Salmonella enterica Remodels the Host Cell Endosomal System for Efficient Intravacuolar Nutrition', *Cell Host Microbe*, 21: 390-402.

Loomis, W. P., M. L. Johnson, A. Brasfield, M. P. Blanc, J. Yi, S. I. Miller, B. T. Cookson, and A. M. Hajjar. 2014. 'Temporal and anatomical host resistance to chronic Salmonella infection is quantitatively dictated by Nramp1 and influenced by host genetic background', *PLoS One*, 9: e111763.

Lotz, M., D. Gutle, S. Walther, S. Menard, C. Bogdan, and M. W. Hornef. 2006. 'Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells', *J Exp Med*, 203: 973-84.

Machado, J. R., D. F. Soave, M. V. da Silva, L. B. de Menezes, R. M. Etchebehere, M. L. Monteiro, M. A. dos Reis, R. R. Correa, and M. R. Celes. 2014. 'Neonatal sepsis and inflammatory mediators', *Mediators Inflamm*, 2014: 269681.

Macierzanka, A., A. R. Mackie, B. H. Bajka, N. M. Rigby, F. Nau, and D. Dupont. 2014. 'Transport of particles in intestinal mucus under simulated infant and adult physiological conditions: impact of mucus structure and extracellular DNA', *PLoS One*, 9: e95274.

MacLennan, C. A., E. N. Gondwe, C. L. Msefula, R. A. Kingsley, N. R. Thomson, S. A. White, M. Goodall, D. J. Pickard, S. M. Graham, G. Dougan, C. A. Hart, M. E. Molyneux, and M. T. Drayson. 2008. 'The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of Salmonella in African children', *J Clin Invest*, 118: 1553-62.

Maier, L., M. Diard, M. E. Sellin, E. S. Chouffane, K. Trautwein-Weidner, B. Periaswamy, E. Slack, T. Dolowschiak, B. Stecher, C. Loverdo, R. R. Regoes, and W. D. Hardt. 2014. 'Granulocytes impose a tight bottleneck upon the gut luminal pathogen population during Salmonella typhimurium colitis', *PLoS Pathog*, 10: e1004557.

Majowicz, S. E., J. Musto, E. Scallan, F. J. Angulo, M. Kirk, S. J. O'Brien, T. F. Jones, A. Fazil, R. M. Hoekstra, and Studies International Collaboration on Enteric Disease 'Burden of Illness. 2010. 'The global burden of nontyphoidal Salmonella gastroenteritis', *Clin Infect Dis*, 50: 882-9.

Malik-Kale, P., S. Winfree, and O. Steele-Mortimer. 2012. 'The bimodal lifestyle of intracellular Salmonella in epithelial cells: replication in the cytosol obscures defects in vacuolar replication', *PLoS One*, 7: e38732.

Marcus, S. L., J. H. Brumell, C. G. Pfeifer, and B. B. Finlay. 2000. 'Salmonella pathogenicity islands: big virulence in small packages', *Microbes Infect*, 2: 145-56.

Marlovits, T. C., and C. E. Stebbins. 2010. 'Type III secretion systems shape up as they ship out', *Curr Opin Microbiol*, 13: 47-52.

Martinoli, C., A. Chiavelli, and M. Rescigno. 2007. 'Entry route of Salmonella typhimurium directs the type of induced immune response', *Immunity*, 27: 975-84.

Mastroeni, P., and A. J. Grant. 2011. 'Spread of Salmonella enterica in the body during systemic infection: unravelling host and pathogen determinants', *Expert Rev Mol Med*, 13: e12.

Mastroeni, P., A. Grant, O. Restif, and D. Maskell. 2009. 'A dynamic view of the spread and intracellular distribution of Salmonella enterica', *Nat Rev Microbiol*, 7: 73-80.

Mastroeni, P., S. Ugrinovic, A. Chandra, C. MacLennan, R. Doffinger, and D. Kumararatne. 2003. 'Resistance and susceptibility to Salmonella infections: lessons from mice and patients with immunodeficiencies', *Reviews in Medical Microbiology*, 14: 53-62.

Mastroeni, P., A. Vazquez-Torres, F. C. Fang, Y. Xu, S. Khan, C. E. Hormaeche, and G. Dougan. 2000. 'Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo', *J Exp Med*, 192: 237-48.

Matsui, H., C. M. Bacot, W. A. Garlington, T. J. Doyle, S. Roberts, and P. A. Gulig. 2001. 'Virulence plasmid-borne spvB and spvC genes can replace the 90-kilobase plasmid in conferring virulence to Salmonella enterica serovar Typhimurium in subcutaneously inoculated mice', *J Bacteriol*, 183: 4652-8.

McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson. 2001. 'Complete genome sequence of Salmonella enterica serovar Typhimurium LT2', *Nature*, 413: 852-6.

McGourty, K., T. L. Thurston, S. A. Matthews, L. Pinaud, L. J. Mota, and D. W. Holden. 2012. 'Salmonella inhibits retrograde trafficking of mannose-6-phosphate receptors and lysosome function', *Science*, 338: 963-7.

McNamee, P. T., and J. A. Smyth. 2000. 'Bacterial chondronecrosis with osteomyelitis ('femoral head necrosis') of broiler chickens: a review', *Avian Pathol*, 29: 477-95.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. 'Food-related illness and death in the United States', *Emerg Infect Dis*, 5: 607-25.

Medzhitov, R. 2007. 'Recognition of microorganisms and activation of the immune response', *Nature*, 449: 819-26.

Menard, S., V. Forster, M. Lotz, D. Gutle, C. U. Duerr, R. L. Gallo, B. Henriques-Normark, K. Putsep, M. Andersson, E. O. Glocker, and M. W. Hornef. 2008. 'Developmental switch of intestinal antimicrobial peptide expression', *J Exp Med*, 205: 183-93.

Meresse, S., O. Steele-Mortimer, B. B. Finlay, and J. P. Gorvel. 1999. 'The rab7 GTPase controls the maturation of Salmonella typhimurium-containing vacuoles in HeLa cells', *EMBO J*, 18: 4394-403.

Mezal, E. H., A. Sabol, M. A. Khan, N. Ali, R. Stefanova, and A. A. Khan. 2014. 'Isolation and molecular characterization of Salmonella enterica serovar Enteritidis from poultry house and clinical samples during 2010', *Food Microbiol*, 38: 67-74.

Miao, E. A., M. Brittnacher, A. Haraga, R. L. Jeng, M. D. Welch, and S. I. Miller. 2003. 'Salmonella effectors translocated across the vacuolar membrane interact with the actin cytoskeleton', *Mol Microbiol*, 48: 401-15.

Miao, E. A., and S. I. Miller. 2000. 'A conserved amino acid sequence directing intracellular type III secretion by Salmonella typhimurium', *Proc Natl Acad Sci U S A*, 97: 7539-44.

Michetti, P., M. J. Mahan, J. M. Slauch, J. J. Mekalanos, and M. R. Neutra. 1992. 'Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium', *Infect Immun*, 60: 1786-92.

Miki, T., R. Goto, M. Fujimoto, N. Okada, and W. D. Hardt. 2017. 'The Bactericidal Lectin RegIIIbeta Prolongs Gut Colonization and Enteropathy in the Streptomycin Mouse Model for Salmonella Diarrhea', *Cell Host Microbe*, 21: 195-207.

Miki, T., and W. D. Hardt. 2013. 'Outer membrane permeabilization is an essential step in the killing of gram-negative bacteria by the lectin RegIIIbeta', *PLoS One*, 8: e69901.

Miller, S. I., A. M. Kukral, and J. J. Mekalanos. 1989. 'A two-component regulatory system (phoP phoQ) controls Salmonella typhimurium virulence', *Proceedings of the National Academy of Sciences of the United States of America*, 86: 5054-58.

Mills, D. M., V. Bajaj, and C. A. Lee. 1995. 'A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome', *Mol Microbiol*, 15: 749-59.

Mogasale, V., S. N. Desai, V. V. Mogasale, J. K. Park, R. L. Ochiai, and T. F. Wierzba. 2014. 'Case fatality rate and length of hospital stay among patients with typhoid intestinal perforation in developing countries: a systematic literature review', *PLoS One*, 9: e93784.

Mogasale, V., B. Maskery, R. L. Ochiai, J. S. Lee, V. V. Mogasale, E. Ramani, Y. E. Kim, J. K. Park, and T. F. Wierzba. 2014. 'Burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment', *Lancet Glob Health*, 2: e570-80.

Monack, D. M., A. Mueller, and S. Falkow. 2004. 'Persistent bacterial infections: the interface of the pathogen and the host immune system', *Nat Rev Microbiol*, 2: 747-65.

Moon, H. W. 1972. 'Vacuolated villous epithelium of the small intestine of young pigs', *Vet Pathol*, 9: 3-21.
Morpeth, S. C., H. O. Ramadhani, and J. A. Crump. 2009. 'Invasive non-Typhi Salmonella disease in Africa', *Clin Infect Dis*, 49: 606-11.
Mota, L. J., A. E. Ramsden, M. Liu, J. D. Castle, and D. W. Holden. 2009. 'SCAMP3 is a component of the Salmonella-induced tubular network and reveals an interaction between bacterial effectors and post-Golgi trafficking', *Cell Microbiol*, 11: 1236-53.
Moxey, P. C., and J. S. Trier. 1979. 'Development of villus absorptive cells in the human fetal small intestine: a morphological and morphometric study', *Anat Rec*, 195: 463-82.
Mueller, C. A., P. Broz, and G. R. Cornelis. 2008. 'The type III secretion system tip complex and translocon', *Mol Microbiol*, 68: 1085-95.
Muller, A. J., P. Kaiser, K. E. Dittmar, T. C. Weber, S. Haueter, K. Endt, P. Songhet, C. Zellweger, M. Kremer, H. J. Fehling, and W. D. Hardt. 2012. 'Salmonella gut invasion involves TTSS-2-dependent epithelial traversal, basolateral exit, and uptake by epithelium-sampling lamina propria phagocytes', *Cell Host Microbe*, 11: 19-32.
Muncan, V., J. Heijmans, S. D. Krasinski, N. V. Buller, M. E. Wildenberg, S. Meisner, M. Radonjic, K. A. Stapleton, W. H. Lamers, I. Biemond, M. A. van den Bergh Weerman, D. O'Carroll, J. C. Hardwick, D. W. Hommes, and G. R. van den Brink. 2011. 'Blimp1 regulates the transition of neonatal to adult intestinal epithelium', *Nat Commun*, 2: 452.
Newton, K., and V. M. Dixit. 2012. 'Signaling in innate immunity and inflammation', *Cold Spring Harb Perspect Biol*, 4.
Noster, J., T. C. Chao, N. Sander, M. Schulte, T. Reuter, N. Hansmeier, and M. Hensel. 2019. 'Proteomics of intracellular Salmonella enterica reveals roles of Salmonella pathogenicity island 2 in metabolism and antioxidant defense', *PLoS Pathog*, 15: e1007741.
Nyirenda, T. S., J. J. Gilchrist, N. A. Feasey, S. J. Glennie, N. Bar-Zeev, M. A. Gordon, C. A. MacLennan, W. L. Mandala, and R. S. Heyderman. 2014. 'Sequential acquisition of T cells and antibodies to nontyphoidal Salmonella in Malawian children', *J Infect Dis*, 210: 56-64.
O'Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campbell, R. P. MacDermott, and S. B. Formal. 1980. 'Genetic control of susceptibility to Salmonella typhimurium in mice: role of the LPS gene', *J Immunol*, 124: 20-4.
Ochman, H., and E. A. Groisman. 1996. 'Distribution of pathogenicity islands in Salmonella spp', *Infect Immun*, 64: 5410-2.
Ochman, H., J. G. Lawrence, and E. A. Groisman. 2000. 'Lateral gene transfer and the nature of bacterial innovation', *Nature*, 405: 299-304.
Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman. 1996. 'Identification of a pathogenicity island required for Salmonella survival in host cells', *Proc Natl Acad Sci U S A*, 93: 7800-4.
Ohlson, M. B., Z. Huang, N. M. Alto, M. P. Blanc, J. E. Dixon, J. Chai, and S. I. Miller. 2008. 'Structure and function of Salmonella SifA indicate that its interactions with SKIP, SseJ, and RhoA family GTPases induce endosomal tubulation', *Cell Host Microbe*, 4: 434-46.
Ong, S. Y., C. B. Pratap, X. Wan, S. Hou, A. Y. Rahman, J. A. Saito, G. Nath, and M. Alam. 2013. 'The Genomic Blueprint of Salmonella enterica subspecies enterica serovar Typhi P-stx-12', *Stand Genomic Sci*, 7: 483-96.
Pabst, O. 2012. 'New concepts in the generation and functions of IgA', *Nat Rev Immunol*, 12: 821-32.
Pabst, O., H. Herbrand, T. Worbs, M. Friedrichsen, S. Yan, M. W. Hoffmann, H. Korner, G. Bernhardt, R. Pabst, and R. Forster. 2005. 'Cryptopatches and isolated lymphoid follicles: dynamic lymphoid tissues dispensable for the generation of intraepithelial lymphocytes', *Eur J Immunol*, 35: 98-107.
Palm, W. 2019. 'Metabolic functions of macropinocytosis', *Philos Trans R Soc Lond B Biol Sci*, 374: 20180285.
Patel, J. C., and J. E. Galan. 2005. 'Manipulation of the host actin cytoskeleton by Salmonella--all in the name of entry', *Curr Opin Microbiol*, 8: 10-5.

Patel, S., D. M. Wall, A. Castillo, and B. A. McCormick. 2019. 'Caspase-3 cleavage of Salmonella type III secreted effector protein SifA is required for localization of functional domains and bacterial dissemination', *Gut Microbes*, 10: 172-87.

Pees, M., W. Rabsch, B. Plenz, A. Fruth, R. Prager, S. Simon, V. Schmidt, S. Munch, and P. Braun. 2013. 'Evidence for the transmission of Salmonella from reptiles to children in Germany, July 2010 to October 2011', *Euro Surveill*, 18.

Peterson, L. W., and D. Artis. 2014. 'Intestinal epithelial cells: regulators of barrier function and immune homeostasis', *Nat Rev Immunol*, 14: 141-53.

Petrie, L., I. E. Selman, M. Grindlay, and H. Thompson. 1977. 'Salmonellosis in young calves due to Salmonella enteritidis', *Vet Rec*, 101: 398-402.

Poh, J., C. Odendall, A. Spanos, C. Boyle, M. Liu, P. Freemont, and D. W. Holden. 2008. 'SteC is a Salmonella kinase required for SPI-2-dependent F-actin remodelling', *Cell Microbiol*, 10: 20-30.

Popoff, M. Y., J. Bockemuhl, and L. L. Gheesling. 2004. 'Supplement 2002 (no. 46) to the Kauffmann-White scheme', *Res Microbiol*, 155: 568-70.

Popp, J., J. Noster, K. Busch, A. Kehl, G. Zur Hellen, and M. Hensel. 2015. 'Role of host cell-derived amino acids in nutrition of intracellular Salmonella enterica', *Infect Immun*, 83: 4466-75.

Prager, R., S. Mirold, E. Tietze, U. Strutz, B. Knuppel, W. Rabsch, W. D. Hardt, and H. Tschape. 2000. 'Prevalence and polymorphism of genes encoding translocated effector proteins among clinical isolates of Salmonella enterica', *Int J Med Microbiol*, 290: 605-17.

Prestinaci, F., P. Pezzotti, and A. Pantosti. 2015. 'Antimicrobial resistance: a global multifaceted phenomenon', *Pathog Glob Health*, 109: 309-18.

Preziosi, M. J., S. M. Kandel, D. G. Guiney, and S. H. Browne. 2012. 'Microbiological analysis of nontyphoidal Salmonella strains causing distinct syndromes of bacteremia or enteritis in HIV/AIDS patients in San Diego, California', *J Clin Microbiol*, 50: 3598-603.

Pui, C. F., W. C. Wong, L. C. Chai, T. Robin, J. Ponniah, M. S. Noor Hidayah, U. Anyi, M. G. Farinazleen, Y. K. Cheah, and R. Son. 2011. 'Review Article Salmonella: A foodborne pathogen', *Int. Food Res. J.*, 18: 465-73.

Quezada, C. M., S. W. Hicks, J. E. Galan, and C. E. Stebbins. 2009. 'A family of Salmonella virulence factors functions as a distinct class of autoregulated E3 ubiquitin ligases', *Proc Natl Acad Sci U S A*, 106: 4864-9.

Rabsch, W., H. L. Andrews, R. A. Kingsley, R. Prager, H. Tschape, L. G. Adams, and A. J. Baumler. 2002. 'Salmonella enterica serotype Typhimurium and its host-adapted variants', *Infect Immun*, 70: 2249-55.

Rakoff-Nahoum, S., Y. Kong, S. H. Kleinstein, S. Subramanian, P. P. Ahern, J. I. Gordon, and R. Medzhitov. 2015. 'Analysis of gene–environment interactions in postnatal development of the mammalian intestine', *Proceedings of the National Academy of Sciences*, 112: 1929-36.

Ramsden, A. E., D. W. Holden, and L. J. Mota. 2007. 'Membrane dynamics and spatial distribution of Salmonella-containing vacuoles', *Trends Microbiol*, 15: 516-24.

Rappl, C., J. Deiwick, and M. Hensel. 2003. 'Acidic pH is required for the functional assembly of the type III secretion system encoded by Salmonella pathogenicity island 2', *FEMS Microbiol Lett*, 226: 363-72.

Remis, N. N., T. Wiwatpanit, A. J. Castiglioni, E. N. Flores, J. A. Cantu, and J. Garcia-Anoveros. 2014. 'Mucolipin co-deficiency causes accelerated endolysosomal vacuolation of enterocytes and failure-to-thrive from birth to weaning', *PLoS Genet*, 10: e1004833.

Renz, H., B. D. Adkins, S. Bartfeld, R. S. Blumberg, D. L. Farber, J. Garssen, P. Ghazal, D. J. Hackam, B. J. Marsland, K. D. McCoy, J. Penders, I. Prinz, V. Verhasselt, E. von Mutius, J. N. Weiser, D. R. Wesemann, and M. W. Hornef. 2018. 'The neonatal window of opportunity-early priming for life', *J Allergy Clin Immunol*, 141: 1212-14.

Renz, H., P. Brandtzaeg, and M. Hornef. 2012. 'The impact of perinatal immune development on mucosal homeostasis and chronic inflammation', *Nat Rev Immunol*, 12: 9-23.

Reynolds, E. S. 1963. 'The use of lead citrate at high pH as an electron-opaque stain in electron microscopy', *J Cell Biol*, 17: 208-12.

Rhen, M., M. Virtanen, and P. H. Makela. 1989. 'Localization by insertion mutagenesis of a virulence-associated region on the Salmonella typhimurium 96 kilobase pair plasmid', *Microb Pathog*, 6: 153-8.

Rice, D. H., T. E. Besser, and D. D. Hancock. 1997. 'Epidemiology and virulence assessment of Salmonella dublin', *Vet Microbiol*, 56: 111-24.

Rivera-Chavez, F., and A. J. Bäumler. 2015. 'The Pyromaniac Inside You: Salmonella Metabolism in the Host Gut', *Annu Rev Microbiol*, 69: 31-48.

Rohde, J. R., A. Breitkreutz, A. Chenal, P. J. Sansonetti, and C. Parsot. 2007. 'Type III secretion effectors of the IpaH family are E3 ubiquitin ligases', *Cell Host Microbe*, 1: 77-83.

Rosa-Ferreira, C., and S. Munro. 2011. 'Arl8 and SKIP act together to link lysosomes to kinesin-1', *Dev Cell*, 21: 1171-8.

Rouch, J. D., A. Scott, N. Y. Lei, R. S. Solorzano-Vargas, J. Wang, E. M. Hanson, M. Kobayashi, M. Lewis, M. G. Stelzner, J. C. Dunn, L. Eckmann, and M. G. Martin. 2016. 'Development of Functional Microfold (M) Cells from Intestinal Stem Cells in Primary Human Enteroids', *PLoS One*, 11: e0148216.

Roudier, C., J. Fierer, and D. G. Guiney. 1992. 'Characterization of translation termination mutations in the spv operon of the Salmonella virulence plasmid pSDL2', *J Bacteriol*, 174: 6418-23.

Roumagnac, P., F. X. Weill, C. Dolecek, S. Baker, S. Brisse, N. T. Chinh, T. A. Le, C. J. Acosta, J. Farrar, G. Dougan, and M. Achtman. 2006. 'Evolutionary history of Salmonella typhi', *Science*, 314: 1301-4.

Royle, M. C., S. Totemeyer, L. C. Alldridge, D. J. Maskell, and C. E. Bryant. 2003. 'Stimulation of Toll-like receptor 4 by lipopolysaccharide during cellular invasion by live Salmonella typhimurium is a critical but not exclusive event leading to macrophage responses', *J Immunol*, 170: 5445-54.

Ruiz-Albert, J., X. J. Yu, C. R. Beuzon, A. N. Blakey, E. E. Galyov, and D. W. Holden. 2002. 'Complementary activities of SseJ and SifA regulate dynamics of the Salmonella typhimurium vacuolar membrane', *Mol Microbiol*, 44: 645-61.

Rytkonen, A., J. Poh, J. Garmendia, C. Boyle, A. Thompson, M. Liu, P. Freemont, J. C. Hinton, and D. W. Holden. 2007. 'SseL, a Salmonella deubiquitinase required for macrophage killing and virulence', *Proc Natl Acad Sci U S A*, 104: 3502-7.

Sabbagh, S. C., C. G. Forest, C. Lepage, J. M. Leclerc, and F. Daigle. 2010. 'So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi', *FEMS Microbiol Lett*, 305: 1-13.

Salcedo, S. P., and D. W. Holden. 2003. 'SseG, a virulence protein that targets Salmonella to the Golgi network', *EMBO J*, 22: 5003-14.

Salcedo, S. P., M. Noursadeghi, J. Cohen, and D. W. Holden. 2001. 'Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages in vivo', *Cell Microbiol*, 3: 587-97.

Salzman, N. H., D. Ghosh, K. M. Huttner, Y. Paterson, and C. L. Bevins. 2003. 'Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin', *Nature*, 422: 522-6.

Sansonetti, P. 2002. 'Host-pathogen interactions: the seduction of molecular cross talk', *Gut*, 50 Suppl 3: III2-8.

Santos, R. L., and A. J. Baumler. 2004. 'Cell tropism of Salmonella enterica', *Int J Med Microbiol*, 294: 225-33.

Santos, R. L., M. Raffatellu, C. L. Bevins, L. G. Adams, C. Tukel, R. M. Tsolis, and A. J. Baumler. 2009. 'Life in the inflamed intestine, Salmonella style', *Trends Microbiol*, 17: 498-506.

Schroeder, B. O. 2019. 'Fight them or feed them: how the intestinal mucus layer manages the gut microbiota', *Gastroenterology report*, 7: 3-12.

Schroeder, N., L. J. Mota, and S. Meresse. 2011. 'Salmonella-induced tubular networks', *Trends Microbiol*, 19: 268-77.

Seifert, J., and W. Sass. 1990. 'Intestinal absorption of macromolecules and small particles', *Dig Dis*, 8: 169-78.

Shea, J. E., C. R. Beuzon, C. Gleeson, R. Mundy, and D. W. Holden. 1999. 'Influence of the Salmonella typhimurium pathogenicity island 2 type III secretion system on bacterial growth in the mouse', *Infection and Immunity*, 67: 213-19.

Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden. 1996. 'Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium', *Proc Natl Acad Sci U S A*, 93: 2593-7.

Sindhwani, A., S. B. Arya, H. Kaur, D. Jagga, A. Tuli, and M. Sharma. 2017. 'Salmonella exploits the host endolysosomal tethering factor HOPS complex to promote its intravacuolar replication', *PLoS Pathog*, 13: e1006700.

Sivick, K. E., N. Arpaia, G. L. Reiner, B. L. Lee, B. R. Russell, and G. M. Barton. 2014. 'Toll-like receptor-deficient mice reveal how innate immune signaling influences Salmonella virulence strategies', *Cell Host Microbe*, 15: 203-13.

Skrzypek, T., S. Szymańczyk, K. Ferenc, W. Kazimierczak, K. Szczepaniak, and R. Zabielski. 2018. 'The contribution of vacuolated foetal-type enterocytes in the process of maturation of the small intestine in piglets. Invited review', *Journal of Animal and Feed Sciences*, 27: 187-201.

Skrzypek, T., J. L. Valverde Piedra, H. Skrzypek, W. Kazimierczak, M. Biernat, and R. Zabielski. 2007. 'Gradual disappearance of vacuolated enterocytes in the small intestine of neonatal piglets', *J Physiol Pharmacol*, 58 Suppl 3: 87-95.

Sojka, W. J., C. Wray, and I. McLaren. 1986. 'A survey of drug resistance in salmonellae isolated from animals in England and Wales in 1982 and 1983', *Br Vet J*, 142: 371-80.

Sojka, W. J., C. Wray, J. Shreeve, and A. J. Benson. 1977. 'Incidence of salmonella infection in animals in England and Wales 1968--1974', *J Hyg (Lond)*, 78: 43-56.

Sojka, W. J., C. Wray, J. E. Shreeve, and J. C. Bell. 1983. 'The incidence of salmonella infection in sheep in England and Wales, 1975 to 1981', *Br Vet J*, 139: 386-92.

Spector, Michael P., and William J. Kenyon. 2012. 'Resistance and survival strategies of Salmonella enterica to environmental stresses', *Food Research International*, 45: 455-81.

Srikanth, C. V., D. M. Wall, A. Maldonado-Contreras, H. Shi, D. Zhou, Z. Demma, K. L. Mumy, and B. A. McCormick. 2010. 'Salmonella pathogenesis and processing of secreted effectors by caspase-3', *Science*, 330: 390-93.

Staley, T. E., and L. J. Bush. 1985. 'Receptor mechanisms of the neonatal intestine and their relationship to immunoglobulin absorption and disease', *J Dairy Sci*, 68: 184-205.

Stecher, B., M. Barthel, M. C. Schlumberger, L. Haberli, W. Rabsch, M. Kremer, and W. D. Hardt. 2008. 'Motility allows S. Typhimurium to benefit from the mucosal defence', *Cell Microbiol*, 10: 1166-80.

Stecher, B., A. J. Macpherson, S. Hapfelmeier, M. Kremer, T. Stallmach, and W. D. Hardt. 2005. 'Comparison of Salmonella enterica serovar Typhimurium colitis in germfree mice and mice pretreated with streptomycin', *Infect Immun*, 73: 3228-41.

Stecher, B., R. Robbiani, A. W. Walker, A. M. Westendorf, M. Barthel, M. Kremer, S. Chaffron, A. J. Macpherson, J. Buer, J. Parkhill, G. Dougan, C. von Mering, and W. D. Hardt. 2007. 'Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota', *PLoS Biol*, 5: 2177-89.

Steeb, B., B. Claudi, N. A. Burton, P. Tienz, A. Schmidt, H. Farhan, A. Maze, and D. Bumann. 2013. 'Parallel exploitation of diverse host nutrients enhances Salmonella virulence', *PLoS Pathog*, 9: e1003301.

Steele-Mortimer, O. 2008. 'The Salmonella-containing vacuole: moving with the times', *Curr Opin Microbiol*, 11: 38-45.

Steele-Mortimer, O., J. H. Brumell, L. A. Knodler, S. Meresse, A. Lopez, and B. B. Finlay. 2002. 'The invasion-associated type III secretion system of Salmonella enterica serovar Typhimurium is necessary for intracellular proliferation and vacuole biogenesis in epithelial cells', *Cell Microbiol*, 4: 43-54.

Steele-Mortimer, O., S. Meresse, J. P. Gorvel, B. H. Toh, and B. B. Finlay. 1999. 'Biogenesis of Salmonella typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway', *Cell Microbiol*, 1: 33-49.

Stein, M. A., K. Y. Leung, M. Zwick, F. Garcia-del Portillo, and B. B. Finlay. 1996. 'Identification of a Salmonella virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells', *Mol Microbiol*, 20: 151-64.

Stockinger, S., C. U. Duerr, M. Fulde, T. Dolowschiak, J. Pott, I. Yang, D. Eibach, F. Backhed, S. Akira, S. Suerbaum, M. Brugman, and M. W. Hornef. 2014. 'TRIF signaling drives homeostatic intestinal epithelial antimicrobial peptide expression', *J Immunol*, 193: 4223-34.

Strawn, L. K., M. D. Danyluk, R. W. Worobo, and M. Wiedmann. 2014. 'Distributions of Salmonella subtypes differ between two U.S. produce-growing regions', *Appl Environ Microbiol*, 80: 3982-91.

Tahoun, A., S. Mahajan, E. Paxton, G. Malterer, D. S. Donaldson, D. Wang, A. Tan, T. L. Gillespie, M. O'Shea, A. J. Roe, D. J. Shaw, D. L. Gally, A. Lengeling, N. A. Mabbott, J. Haas, and A. Mahajan. 2012. 'Salmonella transforms follicle-associated epithelial cells into M cells to promote intestinal invasion', *Cell Host Microbe*, 12: 645-56.

Taitt, C. R., Y. S. Shubin, R. Angel, and F. S. Ligler. 2004. 'Detection of Salmonella enterica serovar typhimurium by using a rapid, array-based immunosensor', *Appl Environ Microbiol*, 70: 152-8.

Takaya, A., M. Suzuki, H. Matsui, T. Tomoyasu, H. Sashinami, A. Nakane, and T. Yamamoto. 2003. 'Lon, a stress-induced ATP-dependent protease, is critically important for systemic Salmonella enterica serovar typhimurium infection of mice', *Infect Immun*, 71: 690-6.

Tanner, J. R., and R. A. Kingsley. 2018. 'Evolution of Salmonella within Hosts', *Trends Microbiol*, 26: 986-98.

Tauxe, R. V. 1997. 'Emerging foodborne diseases: an evolving public health challenge', *Emerg Infect Dis*, 3: 425-34.

Tennant, S. M., C. A. MacLennan, R. Simon, L. B. Martin, and M. I. Khan. 2016. 'Nontyphoidal salmonella disease: Current status of vaccine research and development', *Vaccine*, 34: 2907-10.

Thorne, G. M., and L. M. Corwin. 1975. 'Mutations affecting aromatic amino acid transport in Escherichia coli and Salmonella typhimurium', *J Gen Microbiol*, 90: 203-16.

Torow, N., K. Yu, K. Hassani, J. Freitag, O. Schulz, M. Basic, A. Brennecke, T. Sparwasser, N. Wagner, A. Bleich, M. Lochner, S. Weiss, R. Forster, O. Pabst, and M. W. Hornef. 2015. 'Active suppression of intestinal CD4(+)TCRalphabeta(+) T-lymphocyte maturation during the postnatal period', *Nat Commun*, 6: 7725.

Tsolis, R. M., R. A. Kingsley, S. M. Townsend, T. A. Ficht, L. G. Adams, and A. J. Baumler. 1999. 'Of mice, calves, and men. Comparison of the mouse typhoid model with other Salmonella infections', *Adv Exp Med Biol*, 473: 261-74.

Tsolis, R. M., S. M. Townsend, E. A. Miao, S. I. Miller, T. A. Ficht, L. G. Adams, and A. J. Baumler. 1999. 'Identification of a putative Salmonella enterica serotype typhimurium host range factor with homology to IpaH and YopM by signature-tagged mutagenesis', *Infect Immun*, 67: 6385-93.

Tsolis, R. M., M. N. Xavier, R. L. Santos, and A. J. Baumler. 2011. 'How to become a top model: impact of animal experimentation on human Salmonella disease research', *Infect Immun*, 79: 1806-14.

Tukel, C., M. Raffatellu, A. D. Humphries, R. P. Wilson, H. L. Andrews-Polymenis, T. Gull, J. F. Figueiredo, M. H. Wong, K. S. Michelsen, M. Akcelik, L. G. Adams, and A. J. Baumler. 2005. 'CsgA is a pathogen-associated molecular pattern of Salmonella enterica serotype Typhimurium that is recognized by Toll-like receptor 2', *Mol Microbiol*, 58: 289-304.

Uematsu, S., M. H. Jang, N. Chevrier, Z. Guo, Y. Kumagai, M. Yamamoto, H. Kato, N. Sougawa, H. Matsui, H. Kuwata, H. Hemmi, C. Coban, T. Kawai, K. J. Ishii, O. Takeuchi, M. Miyasaka, K. Takeda, and S. Akira. 2006. 'Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells', *Nat Immunol*, 7: 868-74.

Valdivia, R. H., and S. Falkow. 1997. 'Fluorescence-based isolation of bacterial genes expressed within host cells', *Science*, 277: 2007-11.

van der Velden, A. W., S. W. Lindgren, M. J. Worley, and F. Heffron. 2000. 'Salmonella pathogenicity island 1-independent induction of apoptosis in infected macrophages by Salmonella enterica serotype typhimurium', *Infect Immun*, 68: 5702-9.

Vazquez-Torres, A., J. Jones-Carson, A. J. Baumler, S. Falkow, R. Valdivia, W. Brown, M. Le, R. Berggren, W. T. Parks, and F. C. Fang. 1999. 'Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes', *Nature*, 401: 804-8.

Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulos, and F. C. Fang. 2000. 'Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro', *J Exp Med*, 192: 227-36.

Vazquez-Torres, A., B. A. Vallance, M. A. Bergman, B. B. Finlay, B. T. Cookson, J. Jones-Carson, and F. C. Fang. 2004. 'Toll-like receptor 4 dependence of innate and adaptive immunity to Salmonella: importance of the Kupffer cell network', *J Immunol*, 172: 6202-8.

Vazquez-Torres, A., Y. Xu, J. Jones-Carson, D. W. Holden, S. M. Lucia, M. C. Dinauer, P. Mastroeni, and F. C. Fang. 2000. 'Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase', *Science*, 287: 1655-8.

Veereman-Wauters, G. 1996. 'Neonatal gut development and postnatal adaptation', *European Journal of Pediatrics*, 155: 627-32.

Velge, P., A. Wiedemann, M. Rosselin, N. Abed, Z. Boumart, A. M. Chausse, O. Grepinet, F. Namdari, S. M. Roche, A. Rossignol, and I. Virlogeux-Payant. 2012. 'Multiplicity of Salmonella entry mechanisms, a new paradigm for Salmonella pathogenesis', *Microbiologyopen*, 1: 243-58.

Vidal, S. M., E. Pinner, P. Lepage, S. Gauthier, and P. Gros. 1996. 'Natural resistance to intracellular infections: Nramp1 encodes a membrane phosphoglycoprotein absent in macrophages from susceptible (Nramp1 D169) mouse strains', *J Immunol*, 157: 3559-68.

Vidal, S., M. L. Tremblay, G. Govoni, S. Gauthier, G. Sebastiani, D. Malo, E. Skamene, M. Olivier, S. Jothy, and P. Gros. 1995. 'The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene', *J Exp Med*, 182: 655-66.

Viggiano, D., G. Ianiro, G. Vanella, S. Bibbo, G. Bruno, G. Simeone, and G. Mele. 2015. 'Gut barrier in health and disease: focus on childhood', *Eur Rev Med Pharmacol Sci*, 19: 1077-85.

Voedisch, S., C. Koenecke, S. David, H. Herbrand, R. Forster, M. Rhen, and O. Pabst. 2009. 'Mesenteric lymph nodes confine dendritic cell-mediated dissemination of Salmonella enterica serovar Typhimurium and limit systemic disease in mice', *Infect Immun*, 77: 3170-80.

Vukavic, T. 1984. 'Timing of the gut closure', *J Pediatr Gastroenterol Nutr*, 3: 700-3.

Waldner, L. L., K. D. MacKenzie, W. Koster, and A. P. White. 2012. 'From Exit to Entry: Long-term Survival and Transmission of Salmonella', *Pathogens*, 1: 128-55.

Walker, W. A. 1979. 'Gastrointestinal host defence: importance of gut closure in control of macromolecular transport', *Ciba Found Symp*: 201-19.

Walker, W. A., and K. J. Isselbacher. 1974. 'Uptake and transport of macromolecules by the intestine. Possible role in clinical disorders', *Gastroenterology*, 67: 531-50.

Walthall, K., G. D. Cappon, M. E. Hurtt, and T. Zoetis. 2005. 'Postnatal development of the gastrointestinal system: a species comparison', *Birth Defects Res B Dev Reprod Toxicol*, 74: 132-56.

Wang, Li, Lei Shi, M. J. Alam, Yuhuan Geng, and Lin Li. 2008. 'Specific and rapid detection of foodborne Salmonella by loop-mediated isothermal amplification method', *Food Research International*, 41: 69-74.

Wang, Y., K. Y. Huang, and Y. Huo. 2014. 'Proteomic comparison between Salmonella Typhimurium and Salmonella Typhi', *J Microbiol*, 52: 71-6.

Warsi, O., E. Lundin, U. Lustig, J. Nasvall, and D. I. Andersson. 2019. 'Selection for novel metabolic capabilities in Salmonella enterica', *Evolution*, 73: 990-1000.

Waterman, S. R., and D. W. Holden. 2003. 'Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system', *Cell Microbiol*, 5: 501-11.

Watson, K. G., and D. W. Holden. 2010. 'Dynamics of growth and dissemination of Salmonella in vivo', *Cell Microbiol*, 12: 1389-97.

Watson, P. R., S. M. Paulin, A. P. Bland, P. W. Jones, and T. S. Wallis. 1995. 'Characterization of intestinal invasion by Salmonella typhimurium and Salmonella dublin and effect of a mutation in the invH gene', *Infect Immun*, 63: 2743-54.

Weiss, D. S., B. Raupach, K. Takeda, S. Akira, and A. Zychlinsky. 2004. 'Toll-like receptors are temporally involved in host defense', *J Immunol*, 172: 4463-9.

Wheeler, N. E., P. P. Gardner, and L. Barquist. 2018. 'Machine learning identifies signatures of host adaptation in the bacterial pathogen Salmonella enterica', *PLoS Genet*, 14: e1007333.

Wilcock, B. P., C. H. Armstrong, and H. J. Olander. 1976. 'The significance of the serotype in the clinical and pathological features of naturally occurring porcine salmonellosis', *Can J Comp Med*, 40: 80-8.

Wilson, C. L., A. J. Ouellette, D. P. Satchell, T. Ayabe, Y. S. Lopez-Boado, J. L. Stratman, S. J. Hultgren, L. M. Matrisian, and W. C. Parks. 1999. 'Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense', *Science*, 286: 113-7.

Winfield, M. D., and E. A. Groisman. 2004. 'Evolution and Ecology of Salmonella', *EcoSal Plus*, 1.

Winter, S. E., P. Thiennimitr, M. G. Winter, B. P. Butler, D. L. Huseby, R. W. Crawford, J. M. Russell, C. L. Bevins, L. G. Adams, R. M. Tsolis, J. R. Roth, and A. J. Baumler. 2010. 'Gut inflammation provides a respiratory electron acceptor for Salmonella', *Nature*, 467: 426-9.

Wood, M. W., M. A. Jones, P. R. Watson, S. Hedges, T. S. Wallis, and E. E. Galyov. 1998. 'Identification of a pathogenicity island required for Salmonella enteropathogenicity', *Mol Microbiol*, 29: 883-91.

Woolhouse, M. E., J. P. Webster, E. Domingo, B. Charlesworth, and B. R. Levin. 2002. 'Biological and biomedical implications of the co-evolution of pathogens and their hosts', *Nat Genet*, 32: 569-77.

Worley, M. J., K. H. Ching, and F. Heffron. 2000. 'Salmonella SsrB activates a global regulon of horizontally acquired genes', *Mol Microbiol*, 36: 749-61.

Wray, C., W. J. Sojka, and J. C. Bell. 1981. 'Salmonella infection in horses in England and Wales, 1973 to 1979', *Vet Rec*, 109: 398-401.

Yang, Y. J., M. C. Huang, S. M. Wang, J. J. Wu, C. P. Cheng, and C. C. Liu. 2002. 'Analysis of risk factors for bacteremia in children with nontyphoidal Salmonella gastroenteritis', *Eur J Clin Microbiol Infect Dis*, 21: 290-3.

Zaharik, M. L., B. A. Vallance, J. L. Puente, P. Gros, and B. B. Finlay. 2002. 'Host-pathogen interactions: Host resistance factor Nramp1 up-regulates the expression of Salmonella pathogenicity island-2 virulence genes', *Proc Natl Acad Sci U S A*, 99: 15705-10.

Zarepour, M., K. Bhullar, M. Montero, C. Ma, T. Huang, A. Velcich, L. Xia, and B. A. Vallance. 2013. 'The mucin Muc2 limits pathogen burdens and epithelial barrier dysfunction during Salmonella enterica serovar Typhimurium colitis', *Infect Immun*, 81: 3672-83.

Zhang, K., A. Dupont, N. Torow, F. Gohde, S. Leschner, S. Lienenklaus, S. Weiss, M. M. Brinkmann, M. Kuhnel, M. Hensel, M. Fulde, and M. W. Hornef. 2014. 'Age-dependent enterocyte invasion and microcolony formation by Salmonella', *PLoS Pathog*, 10: e1004385.

Zhang, K., M. Hornef, and M. Fulde. 2015. 'The deadly bite of Salmonella Typhi', *EMBO Rep*, 16: 887-8.

Zhang, K., A. Riba, M. Nietschke, N. Torow, U. Repnik, A. Putz, M. Fulde, A. Dupont, M. Hensel, and M. Hornef. 2018. 'Minimal SPI1-T3SS effector requirement for Salmonella enterocyte invasion and intracellular proliferation in vivo', *PLoS Pathog*, 14: e1006925.

Zhang, Kaiyi, Mathias W. Hornef, and Aline Dupont. 2015. 'The intestinal epithelium as guardian of gut barrier integrity', *Cellular Microbiology*, 17: 1561-69.

6. Appendix

Table 12: Bacterial strains used in this study.

Strains of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) ATCC14028 applied for investigations of SPI-2 effectors in the neonate mouse model are listed. Deletions of specific gene as well as vactor carriage is given. Mutagenesis was performed by Michael Hensel, University of Osnabrück, as described previously (Datsenko and Wanner 2000) followed by transformation of electrocompetent *Salmonella* with pFPV-25.1. Antibiotic resistance cassettes: AMP = ampicillin, CM = chloramphenicol, KAN = kanamycin. Antibiotics were used for growth selection and replica plating at given final concentrations: AMP: 100 µg/ml, CM: 10 µg/ml, KAN: 25 µg/ml.

strain	features	resistance	reference
255	ATCC 14028::pFPV-25.1	AMP	Valdivia *et al.*, 1998, Zhang *et al.*, 2014
268	MvP 643::p3232	AMP + KAN	Wang and Kushner, 1991
293	MvP 643 Δ*sseB*::FRT pFPV-25.1	AMP	this study
313	MvP 497 Δ*sifA*::aph pFPV-25.1	AMP + KAN	Beuzón *et al.*, 2000; Noster *et al.*, 2019
317	MvP 375 Δ*sspH1*::aph pFPV25.1	AMP + KAN	this study
318	MvP 378 Δ*sseI*::aph pFPV25.1	AMP + KAN	this study
319	MvP 1033 Δ*sseL*::aph pFPV-25.1	AMP + KAN	this study
320	MvP 500 *sopD*::aph pFPV-25.1	AMP + KAN	this study
321	MvP 741 Δ*steC*::aph pFPV-25.1	AMP + KAN	this study
322	MvP 498 Δ*pipB2*::aph pFPV25.1	AMP + KAN	Knodler *et al.*, 2003
323	MvP 543 Δ*spvR*::aph pFPV25.1	AMP + KAN	this study
324	MvP 388 Δ*sseF* Δ*sseG* pFPV-25.1	AMP + KAN	Hansen-Wester *et al.*, 2002; Deiwick *et al.*, 2006
326	MvP 376 Δ*sspH2*::aph pFPV-25.1	AMP + KAN	this study
363	MvP 1848 Δ*sscBsseFG*::FRT Δ*sifA*::FRT Δ*sseJ*::aph	AMP + KAN	this study
364	MvP 1847 Δ*sscBsseFG*::FRT Δ*sifA*::FRT Δ*pipB2*::aph	AMP + KAN	this study
368	MvP 1444 Δ*sseB*::kanR pM973	AMP + KAN	this study
8718	MvP 643 Δ*sseB*::FRT Δ*fadL* pFPV-25.1	AMP	Strugnell *et al.*, 2014
9086	MvP 643 Δ*sseB*::FRT Δ*aroP* Δ*pheP* Δ*tyrP* Δ*mtr* Δ*pheA* pFPV-25.1	AMP	Strugnell *et al.*, 2014
9090	MvP 643 Δ*sseB*::FRT Δ*glk* Δ*manXYZ* Δ*ptsG* Δ*uhpT* pFPV-25.1	AMP	Strugnell *et al.*, 2014
9118	MvP 643 Δ*sseB*::FRT Δ*dpp* Δ*opp* Δ*ydgR* Δ*ygdR* Δ*yhiP* pFPV-25.1	AMP	Strugnell *et al.*, 2014

9870 - 9891	ATCC 14028::pFPV-25.1 *proV* tagged (see Table 3)	AMP + CM	Lim *et al.*, 2014
9892 - 9913	MvP 643 Δ*sseB*::FRT pFPV-25.1 *proV* tagged (see Table 3)	AMP + CM	this study

Table 13: Plasmids applied in this study

All plasmids were incorporated to STy by electroporation.

plasmid	features	reference
pFPV-25.1	amp P_{rpsM}::*gfpmut3*	Valdivia *et al.*, 1998
p3232	amp pWSK30::P_{sseA}*sseB*	Hölzer and Hensel, 2010
pM973	amp P_{ssaG}::*gfpmut2*	Hapfelmeier *et al.*, 2005

Table 14: Primer sequences for amplification of *proV* according to (Lim *et al.* 2014)

proV for	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACAGGACGAAGACCGTGAATATGG
proV rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAACTTCGAAGCAGCTCCAG

Declaration

I hereby declare that this thesis is a presentation of my original research work and has been composed solely by myself. Wherever contributions of collaboration partners or others are involved, every effort was made to indicate this clearly, with due reference to the literature or acknowledgement. The study does not contain, in whole or in part, material that has been submitted to obtain any academic degree previously.

Acknowledgement

First and foremost, I would like to thank my supervisor Prof. Dr. Marcus Fulde, who not only dragged me to Berlin and gave me the opportunity to perform my doctoral studies in his group, but also believed in me and my scientific skills anytime I did not. Although I have to admit it almost drove me insane from time to time, you always pushed me to the maximum you thought I could ever reach. Like mine, your expectations were always high, while you simultaneously had constant faith in me. Being given the chance to develop your own ideas, projects and experiments can never be taken for granted, especially not during your PhD studies. You never made me feel like your student, but always equivalent since the very first day we met and I will never forget and always appreciate that your door and mind was always opened to any of my concerns, questions, ideas and demands.

A special thanks to my second supervisor Prof. Dr. Haike Antelmann, who agreed to participate in this project without hesitation, for the time you willingly spent for correction, discussion and open questions.

My supervision group, in particular PD. Dr. Markus Heimesaat, who volunteered as my supervisor for his high interest in my project.

I would like to express my sincere gratitude to Prof. Dr. Mathias Hornef for his encouragement and fruitful contributions to the progress of my doctoral studies. You are a stellar scientist I will always have veneration for and the joy and enthusiasm you feel in research is contagious!

Many collaboration partners contributed to the bulk of data generated for this project. Especially, I would like to thank Dr. Kaiyi Zhang for friendly support on all of my endless questions and problems, Urska Repnik for excellent transelectron microscopic analysis and Prof Dr. Michael Hensel for the bunch of mutagenesis he generated and kindly provided. A special thanks to Prof. Dr. Peter Valentin-Weigand who hosted us back in Hannover and was always open-minded and helpful. Also, my appreciation is due to the entire MiBi lab for an unforgettable time.

All members from the Institute of Microbiology and Epizootics at the Veterinary faculty of the Freie Universität Berlin, but in particular Dr. Karsten Tedin, always ready for more and more discussion on how to proceed and overcome any issues arising. Your mind of ideas always seemed infinite to me. All team members gave us such a warm welcome in Berlin that I felt comfortable and a little less “lost” from the very beginning, particularly due to Heike Görs, the heart and soul of the institute. Furthermore, I would like to express my gratitude to the “AG Awesome”, which provided me a very pleasant working atmosphere, for many laughs every day and for the entire time we could spent together in the office, lab, seminar room and on conferences.

Dr. Dennis Pägelow, sometimes my “working husband”, a sort of guiding big brother, but most of all always a true companion and supporter in a scientific and personal way. Working next to you has always been inspiring and you lifted me up when I had dug myself down to the basement – thank you!

Julia Assmann and Fereshteh Ghazisaeedi, to me, the both of you have been true friends rather than colleagues. You made me laugh when I actually could not even smile, helped me through the hardest times and shared countless lunch dates, evenings, stories, inspiring talks and unforgettable memories with me.

I would also like to thank all of my old and new friends that have accompanied me during my PhD years, especially my dancing company for providing physical compensation for my often-stressful everyday life. Being a piece of the puzzle always helped me keeping my balance in more ways than you can imagine! Sarah Clarenbach for all her lovely, motivating gestures and untold hours of whining she endured, as well as Daniel Bemmerl for proofreading and honest commendation.

Svenja Michel, my bestie, my soulmate and my sister in heart since many years for always supporting me, believing in my success and being as close as one can be, even over the long distance that separated us spatially in the past years. With you by my side, I can reach for the stars. Maximilian Leitner, which I met in the middle of my doctoral studies, who encouraged and grew me especially during the final and most debilitating stage of my doctoral phase, never complained about my priorities and made me feel trusted and loved. Whenever I fall, I know I can count on you to be there to catch me and without the two of you, I would not have made it through the past months. Be sure that I will always carry you with me everywhere I go.

Lastly, I would like to thank my family, most of all my parents who raised me with love and supported me in all my pursuits. Similar to me, you were holding on, even in the toughest times, when I devoted myself to my sorrow. Nothing I have or will ever reach would have been possible without you. I owe you everything.