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The streptomycin mouse model for *Salmonella* diarrhea: functional analysis of the microbiota, the pathogen's virulence factors, and the host's mucosal immune response

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Summary: The mammalian intestine is colonized by a dense microbial community, the microbiota. Homeostatic and symbiotic interactions facilitate the peaceful co-existence between the microbiota and the host, and inhibit colonization by most incoming pathogens ('colonization resistance'). However, if pathogenic intruders overcome colonization resistance, a fierce, innate inflammatory defense can be mounted within hours, the adaptive arm of the immune system is initiated, and the pathogen is fought back. The molecular nature of the homeostatic interactions, the pathogen's ability to overcome colonization resistance, and the triggering of native and adaptive mucosal immune responses are still poorly understood. To study these mechanisms, the streptomycin mouse model for *Salmonella* diarrhea is of great value. Here, we review how S. Typhimurium triggers mucosal immune responses by active (virulence factor elicited) and passive (MyD88-dependent) mechanisms and introduce the S. Typhimurium mutants available for focusing on either response. Interestingly, mucosal defense turns out to be a double-edged sword, limiting pathogen burdens in the gut tissue but enhancing pathogen growth in the gut lumen. This model allows not only studying the molecular pathogenesis of *Salmonella* diarrhea but also is ideally suited for analyzing innate defenses, microbe handling by mucosal phagocytes, adaptive secretory immunoglobulin A responses, probing microbiota function, and homeostatic microbiota–host interactions. Finally, we discuss the general need for defined assay conditions when using animal models for enteric infections and the central importance of littermate controls.

Keywords: *neutrophils, infectious diseases, bacterial, cytokines, lipopolysaccharide, inflammation*

Introduction: bacterial pathogens subverting diarrheal host responses

The mammalian gut represents a highly complex ecosystem featuring a very dense microbial community composed of several hundred different bacterial species. The recent advent of next generation DNA sequencing techniques has provided a census of this consortium (1). Under homeostasis, the

microbiota live in symbiosis with the host by providing nutritional benefits, facilitating the maturation of the intestinal immune system and by inhibiting pathogen growth in the gut ('colonization resistance'). Thus, the ability of pathogens to colonize the gut is toppled by the microbiota and by the host's mucosal immune system. However, the molecular mechanisms conferring protection are still poorly understood. This is explained by the sheer complexity of the system, the lack of techniques for quantifying individual responses in the context of all other responses, and the inability to manipulate the microbiota composition and function in a defined fashion. Recently, progress has been fueled by simplified model systems and the advent of reproducible gut infection models. In particular, the streptomycin mouse model for *Salmonella* diarrhea (2) and the *Citrobacter* mouse model for enteropathogenic *Escherichia coli* infections (3) have been of great importance (*Fig.* 1). Mouse models for *Campylobacter* diarrhea (4, 5) and *C. difficile* 'diarrhea after antibiotic treatment' (6, 7) have also been reported. In these infection models, a well-defined pathogen triggers acute inflammatory responses in a (formerly) undisturbed mucosa. The defined nature of the 'pro-inflammatory insult' and the availability of defined pathogen mutants enabled systematic analyses and represent key advantages over classical mucosal disease models as the dextran sodium sulfate (DSS) model. In the latter model, DSS compromises the mucus layer, and poorly defined molecules/members of the microbiota elicit an innate-response driven gut inflammation (8).

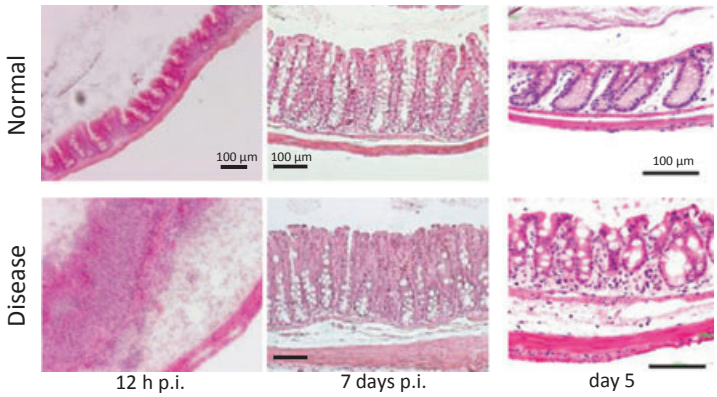
The mucosal infection models provide excellent tools not only for studying the disease triggered by the pathogen's virulence factors but also for analyzing the mechanism(s) of colonization resistance and for functionally probing the innate and adaptive arms of the gut mucosal immune system. Most likely, some interactions will turn out to be specific for the particular pathogen. However, it seems reasonable to assume that most of the mechanisms will be of global importance for understanding pathogen handling by the intestinal mucosa, the mutualism between microbiota and the host and for the function of the intestinal immune system in health and disease.

In this review, we focus on the streptomycin mouse model for S. Typhimurium diarrhea. Originally, this model was based on the treatment of mice with a single oral dose of streptomycin, which transiently depletes the microbiota and thereby disrupts colonization resistance. In these pretreated mice, oral infection with S. Typhimurium leads to massive pathogen growth in the cecum and colon and elicits pronounced acute mucosal inflammation within 6–8 h (2, 9). This model has become very popular, as it provides unsurpassed methods for engineering the pathogen (thus focusing on particular facets of the host response; see below) and because it is particularly robust as indicated by equivalent levels of gut inflammation and disease kinetics in many different laboratories, worldwide.

Streptomycin mouse model for *Salmonella* diarrhea: the basics

Salmonella enterica subspecies 1 serovars Typhimurium and Enteritidis (in short S. Typhimurium and S. Enteritidis) can infect a broad range of host species causing diseases from self-limiting gastroenteritis (e.g. human *Salmonella* diarrhea) to systemic typhoid fever-like infections (10, 11). Classically, typhoid fever-like infections have been studied extensively in mice; while, calves-, primate-, and rabbit-ligated ileal loop models have been instrumental for initial studies of the diarrheal infection. However, for a long time, it has remained enigmatic why mice were generally resistant to *Salmonella* enteropathogenesis. Normally, just 5% of all mice orally infected with S. Typhimurium and/or S. Enteritidis allowed pathogen growth in the gut lumen (i.e. the large intestine) and developed mucosal inflammation in cecum and colon (12, 13). However, if mice are pretreated with a single dose of streptomycin (approximately 20 mg i.g.), every single infected animal shows very high gut colonization levels in the cecum and colon (10^8–10^{10} cfu/g stool) and develops large intestinal inflammation within the first day after oral infection, thus establishing the 'streptomycin mouse model' for *Salmonella* diarrhea (2). Also, the consistency of the stool softens and stool water content increases to some extent (2, 14). Later work established that this model can be extended to numerous other *S. enterica* serovars, including not only the broadly used Typhimurium strains (SL1344, ATCC14028, DT104) and Enteritidis strains (125109, 5496/98, 832/9 9), but also Dublin (SARB13, SD2229, and SD3246), Pullorum (X3543 and 449/87), and Gallinarum (287/91) (15, 16). Thus, the streptomycin mouse model seems suitable for studying enteropathogenesis of many (if not all), serovars known to cause mucosal disease in humans. However, most mechanistic studies have focused on Typhimurium strains SL1344 (17) and ATCC14028 (ATCC).

Detailed analyses established that streptomycin treatment leads to a transient clearance of the microbiota (>10-fold reduced in density and composition) (18) (*Fig.* 2*A*) reliably suppressing colonization resistance for approximately 24–48 h (2, 19–23). In principle, the same effect can also be achieved with other antibiotics like ampicillin, kanamycin, or

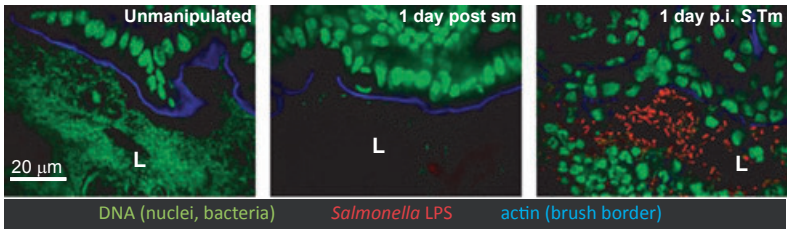


	***Salmonella* colitis model**	***Citrobacter* colitis model**	**DSS colitis model**
Acute colitis Model	Intragastric infection 24 h after 20 mg oral streptomycin (Barthel 2003)	Intragastric infection 10^8 cfu *C. rodentium*	2–4% Dextran-sulfate sodium in drinking water for 5 days (Okayasu 1990)
Time to onset of symptoms	8–12 h postinfection	7 days postinfection (variable)	2–10 days (variable)
Recovery	21 days postinfection (in C57Bl/6 mice infected with attenuated *Salmonella* strains; variable); In slc11a1$^{+/+}$ mice, wt S. Typhimurium causes a chronic infection lasting for months	21 days postinfection (variable)	10–15 days (variable)
Localization of pathology	Starts in cecum; extends to colon within 1–3 days	Colon	Colon
Pathology	Neutrophil infiltrate, submucosal edema, goblet cell depletion, epithelial disruption, crypt abscesses (Barthel 2003)	Colonic crypt hyperplasia with suppurative inflammation, epithelial necrosis, neutrophil infiltrate, goblet cell depletion (Luperchio and Schauer 2001)	Neutrophil infiltrate, submucosal edema, epithelial disruption, crypt abscesses (Dieleman LA 1998)
Mechanism of triggering	Direct triggering by bacterial virulence factors and innate immune responses to bacterial mucosa invasion	Bacterial virulence factors and pathogen attaching and effacing	Disruption of mucus barrier (Johansson 2010) , translocation of commensal bacteria
Biohazard safety measures	BL2 for Wild type *S.* Tm; BL1 for attenuated strains (*aroA*, *sseD)*	BL2	None

Fig. 1. Mouse models for gut inflammation. The images depicting *S.* Typhimurium colitis were taken from HE-stained tissue sections mice at 12 h postinfection. *Citrobacter rodentium* and dextran sodium sulfate (DSS) colitis images are reproduced with permission from Johansson *et al.* and Bergstrom *et al.* (8, 134). The pathology was described previously in detail (2, 3, 235, 236).

vancomycin (24, 25, reviewed in 26). Studies assessing the effect of antibiotic treatment on the mucosal immune system have shown that acute microbiota depletion is also accompanied by slight changes in mucosal immune homeostasis. Daily gavage with a cocktail of ampicillin, gentamicin, metronidazole, neomycin, and vancomycin over 10 days reduced interferon-γ (IFNγ) and interleukin-17 (IL-17) production by *lamina propria* CD4$^+$ T-cells (27). Treat-

A



B

Chemokines				Other inflamm. related genes	
CCL-motive	Induction	Cytokines	Induction	Adamts4	10–100
Ccl2	100–1000	IFN γ	100–1000	Areg	10–100
Ccl4	100–1000	IL-1β	100–1000	Casp1	< 10
Ccl7	100–1000	IL-1r1	< 10	Clec4d	100–1000
Ccl11	10–100	IL-1m	100–1000	Cybb	< 10
Ccl22	10–100	IL-12b	10–100	Gbp2	100–1000
		IL-17a	> 1000	Gem	10–100
CXCL-motive		IL-17f	> 1000	Iigp1	100–1000
Cxcl1	> 1000	IL-22	> 1000	Irg1	> 1000
Cxcl2	> 1000	IL-23a	< 10	Krt36	100–1000
Cxcl5	> 1000	TNF	10–100	Mfsd2	100–1000
Cxcl9	100–1000			Mmp13	100–1000
Cxcl10	100–1000			Nos2	10–100
Cxcl11	100–1000			Procr	10–100
Cxcl16	> 1000			Ptx3	100–1000

C

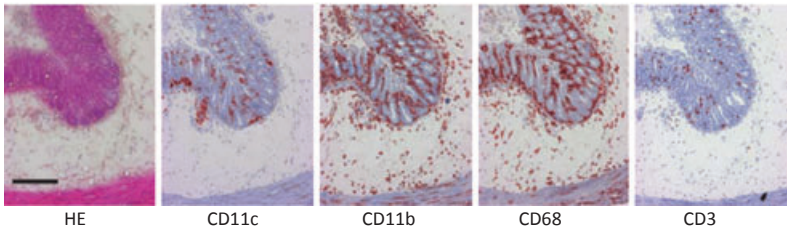


***Fig. 2.* Streptomycin mouse model for *Salmonella* diarrhea.** (A) Gavage with 20 mg streptomycin eliminates more than 90% of the resident microbiota and alleviates colonization resistance. Reproduced with permission from (18). (B) Mucosal gene expression triggered within 12 h after infection with wt S. Typhimurium (138). (C) The mucosal response includes tissue infiltration by monocyte (CD11c, CD11b, CD68 = activation marker), PMN and T-cell (CD3) populations. Reproduced with permission from Hapfelmeir *et al.* (107).

ment with vancomycin, neomycin, and metronidazole reduced expression of the anti-microbial lectin RegIIIγ and conversely led to reduced killing of vancomycin-resistant *Enterococcus faecium* (28). Furthermore, decreased thickness of the colonic mucus layer was shown in metronidazole-treated mice, and this correlated with increased severity of *Citrobacter rodentium*-induced colitis (29). And finally, different classes of antibiotics induced differential microbiota killing and some correlations have been reported between the microbiota composition and susceptibility to infection (30). In any case, with respect to the S. Typhimurium diarrhea model, the most important consequence of antibiotic treatment seems to be the transient disruption of colonization resistance.

After orogastric infection of streptomycin-treated mice, S. Typhimurium grows up to high densities (>10^7 cfu/g stool) within 4–6 h, in particular in the large intestine (2). The final density of the S. Typhimurium population is independent of the size of the inoculum (e.g. 100 cfu or 5×10^7 cfu) and the elicitation of disease (albeit with minor impact on disease kinetics), making the model very robust toward slight changes in the size of the applied inoculum. Furthermore, pathogen densities and the amounts of pathogen-derived molecules (PAMPs) are equivalent between wild-type strains and avirulent mutants, at least during the first 2 days postinfection (2, 18, 31–33), allowing the investigation of specific contributions of the virulence factors or host responses of interest. This is an important advantage over the *Citrobacter* model where virulence factor expression, disease intensity, and gut luminal pathogen loads show a pronounced and complex inter-dependence (34).

Six to ten hours after oral or intragastric inoculation, the pathogen has infected and invaded the intestinal mucosa, and massive inflammation is mounted in the cecal and colonic mucosa. Pathogen invasion is observed in the organized structures of the gut-associated immune system (i.e. the Peyer's patches and the cecal patch; similar to typhoid model) and in the absorptive mucosa. The latter is specific for streptomycin-treated mice. For mounting acute mucosal defense, infection of the Peyer's patches and the cecal patch seems dispensable, as disease parameters were not affected in LTβR$^{-/-}$ mice lacking these structures (2). Therefore, subsequent studies of the patho-mechanisms eliciting enteropathy have focused on the absorptive mucosa.

The basic virulence factors of the pathogen required for eliciting disease, the pathology of the infected gut tissue (mucosal edema, infiltration by polymorphonuclear granulocytes, monocytic phagocytes, T-cells; epithelial damage, reduced numbers of mucus-loaded goblet cells), and the pro-inflammatory gene expression profile of the infected mucosa (*Fig.* 2*B*,*C*) are equivalent between the primate, the bovine (i.e. 'traditional' models for *Salmonella* enteropathogenesis), and the streptomycin mouse model. However, some differences exist with respect to the intestinal location of the most severe disease symptoms. In calves and humans, the small and the large intestine can be affected (10), while streptomycin-treated mice display enteropathy of the cecum and the colon (100% of infected mice). The terminal ileum is affected in <10% of all mice. The reasons for host-specific site preferences will be an interesting topic for future research. Nevertheless, the streptomycin mouse model [i.e. cecum inflammation (2)] has emerged as a very robust and important tool to study *S.* Typhimurium-induced enteropathy. Excellent genetic tools for engineering mutant pathogen strains, broad availability of knockout/transgenic mice, elaborate methods for studying pathogen and host responses, and the advent of next generation sequencing technologies have opened the door to gain insights into molecular and cellular mechanisms driving all stages of the diarrheal *S.* Typhimurium infection.

The mechanism(s) eliciting acute *S.* Typhimurium colitis

Work from the past few years has established that *S.* Typhimurium elicits acute mucosal inflammation via at least two different pathways in parallel. In the 'classical' pathway, the pathogen mainly employs specific virulence factors for triggering pro-inflammatory responses, while the 'alternative' pathway elicits mucosal inflammation by pathogen recognition via the innate immune system. These initial signals are amplified, leading to a tissue-wide inflammation which can last anywhere from a few days to months, depending on the experimental setup, used (2, 24, 31–33, 35) (see below).

The triggering of inflammation via the classical and the alternative pathway depends on the two main virulence factors of *S.* Typhimurium, namely type-III secretion systems TTSS-1 and TTSS-2, which are encoded in the *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 (2, 31). In wildtype mice, both pathways seem to contribute in parallel. However, the use of site-specific *S.* Typhimurium mutants allows studying both mechanisms separately.

The classical pathway

In the classical pathway, *S.* Typhimurium employs the TTSS-1 to invade the gut epithelium, entering the *lamina propria* and eliciting gut inflammation within the first 6–10 h after infection (*Fig.* 3). *S.* Typhimurium mutants with a disrupted TTSS-2 (but an intact TTSS-1; e.g. M556, SL1344 *sseD::aphT*) are restricted to this mechanism (36, 37). The pro-inflammatory signals are thought to be triggered actively by TTSS-1 effector proteins while innate, MyD88- (or Nod1/2-, see below) dependent pathogen recognition seems to play a less prominent role, as indicated by the observation that *S.* Typhimurium enteropathy is just slightly, but significantly, reduced in MyD88$^{-/-}$ mice (36, 38).

TTSS-1 is expressed when *S.* Typhimurium grows in the gut lumen (39). Chemotaxis drives the pathogen toward the gut epithelium (40, 41), and the pathogen binds to gut epithelial cells (enterocytes), presumably via flagella, adhesins like type-I fimbriae or the SiiE non-fimbrial adhesin, and the TTSS-1 itself (37, 41–47). Upon binding, TTSS-1 injects a cocktail of at least 14 different effector proteins into the host cell cytosol where they manipulate signaling. Host cell manipulation by *S.* Typhimurium effector proteins has been studied extensively in tissue culture models. We are just beginning to understand how these effectors contribute to disease *in vivo* (*Table* 1). Thus far, the TTSS-1 effector proteins SipA, SopE, and to a lesser extent SopE2 were proven necessary for driving mucosal invasion and gut inflammation in the streptomycin mouse model (37, 48–50).

SipA (SspA)

In vitro work suggests that SipA belongs to the first wave of TTSS-1 effector proteins, arriving in the host cell as early as 10–90 s after pathogen binding to the host cell (51, 52). SipA has at least four different domains, an N-terminal domain facilitating injection via TTSS-1, two domains affecting SipA

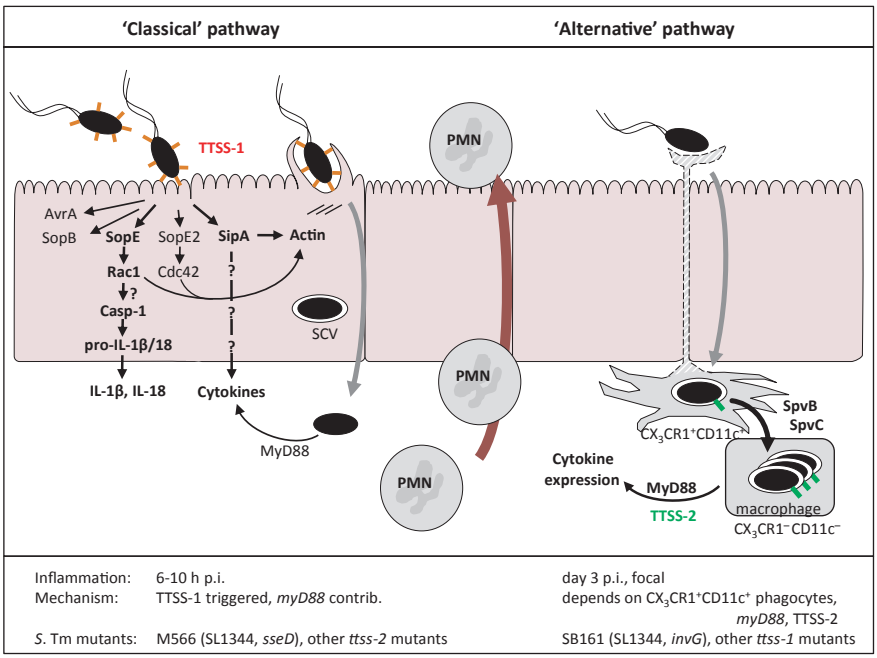


***Fig. 3.* Mechanism(s) eliciting mucosal inflammation in the streptomycin mouse model.**

***Table 1.* SPI-1 effectors: enzymatic activity and molecular function**

Effector	Biochemical activity	Phenotype/function	References
AvrA	Acetyltransferase	Stabilization of IκB, inhibition of NF-κB signaling and apoptosis	(85)
SipA (SspA)	Actin binding	Inhibition of actin depolymerization, membrane ruffling, initiation of inflammation *in vivo*	(37, 54, 55, 237)
SipB (SspB)	Cholesterol binding	TTSS-1 translocon formation	(238–240)
SipC (SspC)	Actin binding	TTSS-1 translocon formation, actin stabilization, and nucleation	(238, 240)
SipD (SspD)		Effector secretion regulation	(241)
SlrP	Putative ubiquitin ligase	Not known	(242)
SopA	Ubiquitin E3 ligase activity	Induction of inflammation *in vivo*	(243–246)
SopB (SigD)	Phosphatidylinositol phosphatase	Invasion, modulation of vesicle trafficking, SCV maturation	(97, 246–249)
SopD	Not known	Systemic replication *in vivo*, invasion into polarized cells	(63, 246, 250, 251)
SopE	Guanine-nucleotide exchange factor for Rac1 and CDC42	Actin-remodeling *in vitro*, caspase-1 activation and induction of inflammation *in vivo*, membrane ruffling	(37, 48, 64, 67, 246)
SopE2	Guanine-nucleotide exchange factor for CDC42	Actin-remodeling *in vitro*, induction of inflammation, membrane ruffling	(37, 63, 81, 83, 246)
SptP	GTPase activating protein for Rac1 and CDC42	Reversal of actin remodeling, inhibition of MAPK signaling and IL-8 secretion	(246, 252–255)
SspH1	Not known	Inhibition of NF-κB signaling	(246, 253, 256)
SteA (STM 1583)	Not known	Not known	(246, 257)
SteB (STM 1629)	Putative dipicolinate reductase	Not known	(257)

localization within the host cell, and a C-terminal domain which binds to actin and acts as an actin depolymerization inhibitor (53–60). The capacity to manipulate the host cell's actin cytoskeleton explains why SipA can enhance host cell invasion (61, 62). Similarly, *S.* Typhimurium requires SipA for efficient invasion into the cecal mucosa and to elicit mucosal inflammation in the streptomycin mouse model and in bovine infection models alike (37, 48–50, 63). This work was enabled by *S.* Typhimurium *sipA* mutants (e.g. M715 *sseD*::*aphT* Δ*sipA*) and by mutants lacking *sopE*, *sopE2* and/or other TTSS-1 effector proteins, but retaining *sipA* (e.g. M716 *sseD*::*aphT* Δ*sopB* Δ*sopE sopE2*::pM218) (37, 48). However, further work will be required to identify the step of the tissue invasion process enhanced by SipA (e.g. enterocyte docking, host cell invasion, epithelial disruption, etc.) and how this initiates a pro-inflammatory response.

SopE

SopE is a potent guanine-nucleotide exchange factor (GEF) activating Rho GTPases such as Rac1 and Cdc42 proteins (64–67). *In vitro*, this triggers a signaling cascade activating WASP/-WAVE complexes and actin polymerization by the Arp2/3 complex, thus initiating cytoskeletal rearrangements and the formation of membrane ruffles which engulf the bacterium and enable invasion (47, 64, 68). This explained why SopE can contribute to mucosa invasion *in vivo* (37, 48). Only recently, it was discovered that SopE also elicits a pro-inflammatory signal (*Fig. 4A*). In stromal cells (presumably enterocytes) of the infected gut, SopE-mediated Rac1/Cdc42 activation triggers a signaling cascade, which activates caspase-1 and thereby the maturation/release of potent cytokines of the IL-1 family, namely IL-1 and IL-18 (48, 67) (*Fig. 4A,B,C*). This response might be of broad relevance to bacterial gut infection, as other bacterial enteropathogens like *Shigella flexneri*, *C. rodentium*, *Chromobacter violaceum*, and enteropathogenic *E. coli* strains are known to express WxxxE family TTSS effector proteins which are functional homologs of SopE (69–71, reviewed in 72).

SopE-mediated triggering of caspase-1/IL-1 responses can be regarded as an adaptation to a particular pathogenic lifestyle. In *S.* Typhimurium strain SL1344 and the epidemic strain DT204, SopE is encoded by the P2-like prophage SopEΦ (73–75). Other *S.* Typhimurium strains like ATCC14028 lack this prophage and do not express *sopE* (73, 76). This finding suggested that shuffling of the effector protein repertoire by lysogenic conversion with phages like SopEΦ may contribute to the *Salmonella* adaptation to new hosts and the emergence of new epidemic strains (73, 77, 78, reviewed in 79). In line with this hypothesis, the lysogenic conversion of ATCC14028 by SopEΦ led to a slight but significant increase in the enteropathogenic potential of the pathogen (80).

Conceptually, the Rac1/caspase-1/IL-1 cascade is quite interesting, as it generates pro-inflammatory signals by proteolytic activation of inactive precursors. This response should be very fast, and initiation would not require host-cellular gene expression. It is tempting to speculate that it may repre-

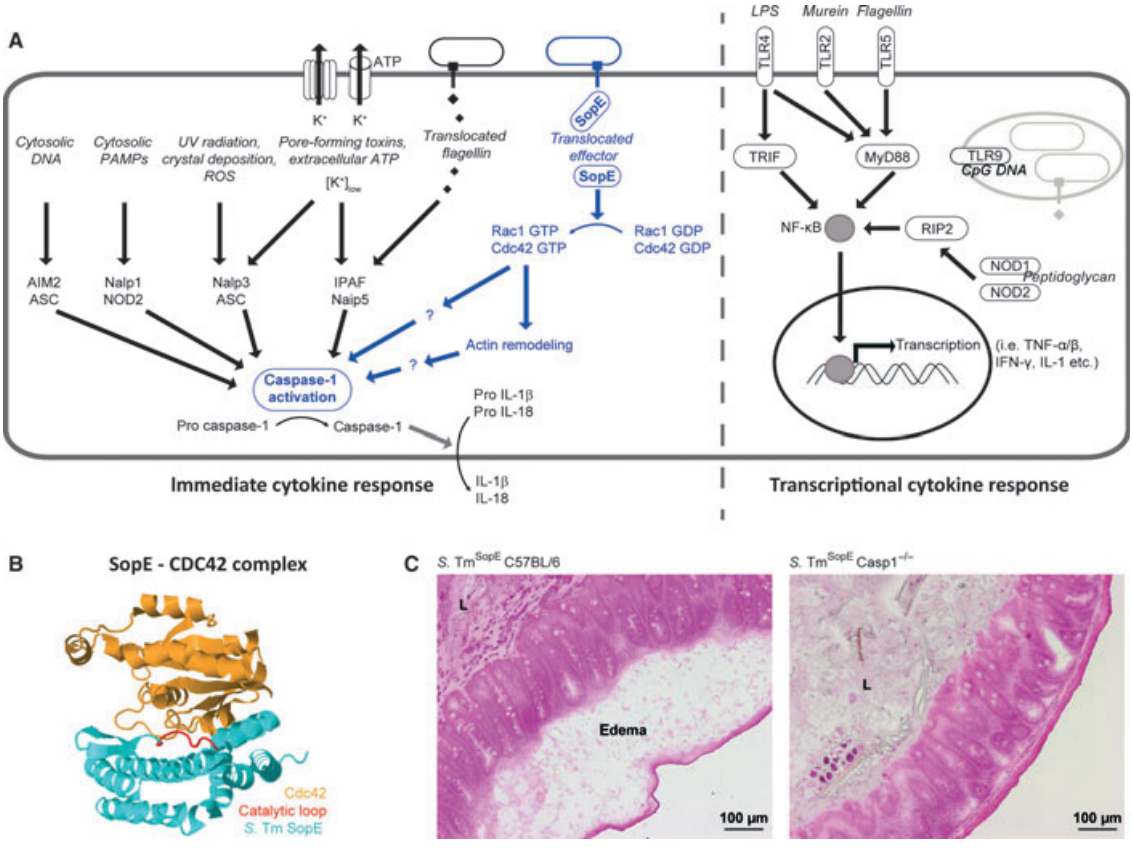


Fig. 4. Triggering of pro-inflammatory responses by SopE. (A) SopE-mediated activation of Rac1 and Cdc42 triggers a signaling cascade which activates the caspase-1/IL-1/IL-18 arm of the innate immune defense. (B) Structure of the SopE–Cdc42 complex (66). Reproduced with permission (72). (C) SopE elicits gut inflammation in wt mice, but not in littermates lacking caspase-1 (Day 2 postinfection with M717 *sseD*::*aphT* Δ*sopB sopE2*::*pM218* Δ*sipA*). L, gut lumen. Tissues were from Muller *et al.* (48).

sent a general 'alert' mechanism, allowing enterocytes to initiate defenses in response to damage inflicted by toxins, chemicals, or pathogens. However, a number of key questions remain to be answered: what is the signaling cascade linking Rac1/Cdc42 to caspase-1 activation? Which other inflammasome components are involved? Which IL-1 family members are activated initially by the infected enterocyte, and who is responsible for the amplification of this initial signal? *S.* Typhimurium mutants lacking all major TTSS-1 effectors except SopE (e.g. M717 *sseD*::*aphT* Δ*sopB sopE2*::*pM218* Δ*sipA*) focus the pro-inflammatory response on the Rac1/caspase-1/IL-1 cascade and enable specific investigation of these questions (37, 48, 67).

SopE2

SopE2 is 69% identical to SopE and is expressed by all known *Salmonella* strains (81, 82). However, in contrast to SopE, which is an efficient GEF for Rac1 and Cdc42 and Rac1, SopE2 has a target protein preference for Cdc42 but not Rac1 (83). This might be of relevance, as Rac1 activation seems to be more important for caspase-1 activation than the activation of Cdc42 (48). Furthermore, the expression levels of SopE2 (approximately 250/bacterium) seem to be approximately 10-fold lower than those of SopE (approximately 2000–6000/bacterium) (Duss I and Hardt WD, unpublished data). These observations might explain why SopE2 itself is a weak activator of host cell invasion *in vitro* (62, 81, 82) and makes just a small contribution to gut inflammation *in vivo* (37). Nevertheless, it is quite clear that SopE2 contributes to the classical pathway by *S.* Typhimurium SL1344. It will be interesting to see if SopE2 (or point mutants with enhanced Rac1 activation) might have a more pronounced function in other Typhimurium strains or other *S. enterica* serovars.

AvrA

AvrA is a TTSS-1 effector protein of the *Yersinia* YopP/YopJ family, which is present in just a subset of all *S. enterica* serovars and strains (84). It acts as an acetyltransferase which chemically modifies mitogen-activated protein kinase kinases and thereby inactivates c-Jun N-terminal kinase (JNK) signaling pathways (85–87). In tissue culture, inhibition of JNK signaling pathways prevents pro-inflammatory responses as well as pro-apoptotic caspase-3 activation (85, 86). It is still debated whether AvrA can directly stabilize IκB and therefore inhibit NF-κB signaling, another important pathway leading to immune activation. In some studies, *avrA*-dependent inhibition of epithelial cell death has been observed at early stages of the infection, in particular if using TTSS-1 overexpressing strain backgrounds (86, 88). However, in the wildtype *S.* Typhimurium SL1344 background, *avrA* deficiency does not affect the overt pathology (88, Müller A and Hardt WD, unpublished data), while slight changes in mucosal gene induction were observed in one study (87). Furthermore, *avrA*-dependent inhibition of enterocyte signaling pathways might be restricted to certain *Salmonella* strains and might be modulated by strain-specific differences in *avrA* gene expression (89, 90). In conclusion, AvrA seems to be another TTSS-1 effector protein modulating the pathogen–host interaction in a strain-specific fashion and might contribute to the adaptation to new hosts and/or the emergence of epidemic strains.

At first glance it may seem counter-intuitive that *S.* Typhimurium deploys pro- (e.g. SipA, SopE, SopE2) and anti-inflammatory effector proteins (e.g. AvrA) at the same time. The recent discovery of 'neighboring cell activation' (also termed 'bystander' activation) might resolve this conundrum (91, 92, discussed in 93, 94). At least in tissue culture, pro-inflammatory gene expression was observed not only in epithelial cells directly infected by *L. monocytogenes*, *S. flexneri*, or *S.* Typhimurium, but also in non-infected neighboring cells. In the case of *S. flexneri*, a dedicated TTSS effector protein (OspF) was inhibiting the pro-inflammatory response in the pathogen-harboring cell, while neighboring cell responses remained unmodulated (91). Thus, from the perspective of the pathogen, the inhibitory activity of AvrA might prolong the survival of the infected host cell and thereby enhance pathogen survival, while inflammation is still triggered by neighboring cell activation and by the SopE/Rac1/caspase-1/IL-1 axis, which relies on preformed precursor protein activation. Conversely, from the perspective of the host, neighboring cell activation might ensure efficient pro-inflammatory cytokine expression by the infected epithelium, even if the infected cell is manipulated by the pathogen. Either way, neighboring cell activation would provide a plausible explanation for the puzzling observation that several mucosal pathogens notorious for eliciting mucosal inflammation, do express potent inhibitors of NF-κB, JNK, ERK, and p38 signaling like OspF and OspG from *S. flexneri* or AvrA from *S.* Typhimurium (86, 95). Clearly, the streptomycin mouse model provides an excellent experimental system for addressing these hypotheses.

SopB/SigD

SopB (SigD) is a TTSS-1 effector protein with phosphatidylinositol phosphatase activity (96, 97). In tissue culture assays, this activity contributes to host cell invasion, presumably by a

RhoA/myosin II-dependent mechanism (98), maturation of the *Salmonella*-containing vacuole (SCV) and host cell viability (99–103). In bovine gut infection models, *sopB* contributes to virulence (63, 96, 104). In contrast, this effect seems less pronounced in the streptomycin mouse model. In wildtype C57BL/6 mice, disease parameters do not differ significantly between wildtype *S.* Typhimurium and isogenic *sopB* mutants (37). However, in wildtype 129S and *akt*$^{-/-}$ mice, *sopB* mutants might have altered virulence phenotypes (105). The underlying mechanisms may be an interesting topic for future research.

In addition, TTSS-1 injects a number of other effector proteins into infected host cells. Tissue culture work and biochemical analyses have revealed potential host cell targets manipulated by these effector proteins (106). However, whether and how they contribute to disease remains to be established. Again, the streptomycin mouse model offers an excellent experimental system. As illustrated above (e.g. for SopE), the combination of *S.* Typhimurium mutants with appropriate knockout mice will allow focusing the infection process to particular disease mechanisms. This will generate new insights into general strategies employed by the mucosal immune system for defense against pathogens and homeostasis with the normal microbiota.

Alternative pathway

S. Typhimurium mutants lacking a functional TTSS-1 elicit gut inflammation via the 'alternative' pathway. This pathway can be studied with mutants like SB161 (SL1344, Δ*invG*) and overt enteropathy is visible by 72 h postinfection (31) (*Fig.* 3). The cecal and colonic pathology shows distinct 'inflammatory foci' harboring significant numbers of bacteria, separated by mucosal areas showing no signs of infection (36). It is conceivable that this pathway of mucosa infection may represent a general mechanism for microbe handling by the mucosal immune system.

In general, TTSS-2 is only induced after *S.* Typhimurium has entered into a host cell. In the alternative pathway, *S.* Typhimurium is thought to penetrate the epithelial barrier in a 'DC-dependent' process bypassing the need for epithelial cell invasion via TTSS-1 (107). In line with this notion, Δ*invG* is very poor in infecting enterocytes of the cecal mucosa *in vivo* (36, 107). However, the mechanism of epithelial penetration via the alternative pathway is still not entirely clear. TTSS-1-independent transport into cryptopatches, as observed in the mouse typhoid fever model (108), would be conceivable. Alternatively, trans-epithelial transport via dendritic cell extensions might occur (109–111). So far, this issue could not be resolved, because the low number of entry events (as low as 50 events in 3 days) has precluded the direct observation of this step of the infection in the streptomycin mouse model.

In any case, Δ*invG* requires CD11c^{+}CX3CR1^{+} monocytic phagocytes (formerly known as mucosal dendritic cells) to colonize the cecal *lamina propria*. Moreover, these cells represent the initial replicative niche for the pathogen after crossing the epithelium (107). *S.* Typhimurium requires TTSS-2 for persistence and growth within these cells, as double mutants with defective TTSS-1 and -2 systems are avirulent. Therefore, the alternative pathway is also termed 'TTSS-2 dependent'. Within 2–3 days postinfection, the pathogen relocates into CX3CR1^{-} phagocytes, grows up to densities of approximately 10^5–10^6 cfu/g of cecal tissue, and elicits mucosal inflammation in a MyD88-dependent fashion (36, 107). Recent data from systemic *S.* Typhimurium infection in the mouse typhoid fever model indicate that the TTSS-2 dependency of inflammation via the alternative pathway might result from the interplay between innate immunity and the pathogen's gene expression (112): in the absence of TLR2, TLR4, and TLR9, mouse macrophages were severely attenuated in pathogen handling. Strikingly, *S.* Typhimurium failed to upregulate key TTSS-2 genes in these macrophages. The failure to induce important TTSS-2 genes ultimately led to impaired intracellular replication in TRL2/4/9-deficient macrophages. Thus, innate immune defense within the host's macrophages may represent a key environmental cue for *Salmonella* virulence factor gene expression. However, *S.* Typhimurium strains harboring reporter plasmids expressing *gfp* from a TTSS-2 promoter, do express GFP during growth within *myD88*$^{-/-}$ *lamina propria* phagocytes (36, 107). This suggests that MyD88 dependency of TTSS-2 expression might differ between different host organs and respective cell types or the different stages of the infection. Alternatively, TRIF-dependent TLR4 signaling may be sufficient to induce TTSS-2 gene expression. In conclusion, the MyD88-dependency of the alternative pathway might be attributable to the detection of high pathogen loads in the infected mucosa and/or the MyD88-dependent expression of the full virulence factor repertoire of the pathogen. Future work will have to resolve this issue.

SpvB and SpvC have also been implicated in the alternative pathway. The *S.* Typhimurium *spv* operon is encoded on a large virulence plasmid present within most subspecies I serovars of *S. enterica* (113). SpvB is an ADP-ribosyltransferase capable of disrupting actin function and contributing to cytotoxicity in macrophages (114, 115). SpvC is a

phosphothreonine lyase interfering with the MAPK pathway (116, 117). This might modulate innate immune responses, like IL-8 and TNF-α gene expression (117, 118). The *spv* operon is essential for full systemic virulence (117, 119, 120). Recently, *spvB* and *spvC* have been implicated in the alternative pathway, i.e. the transition from $CD11c^+$ into $CD11c^-$ phagocytes of the infected mucosa (121).

In any case, *S.* Typhimurium mutants infecting via the alternative pathway represent an excellent tool for probing the function of mucosal phagocyte populations, i.e. $CD11c^+CX3CR1^+$ monocytic phagocytes (107, 122). This may be of great interest not only for infection biology but also for understanding microbiota handling by the mucosal immune system.

In the wildtype *S.* Typhimurium infection, the classical and the alternative pathway seem to act in concert. Tissue invasion occurs via active, TTSS-1 dependent invasion and probably also via TTSS-1 independent mechanisms. And the initiation of mucosal inflammation is driven actively by bacterial virulence factors, and passively by innate immune responses toward microbe associated molecular patterns. On top of this, additional virulence factors (TTSS-1/2 dependent and independent) enhance the efficiency of mucosa invasion and modulate host-cellular responses. The use of appropriate *S.* Typhimurium mutants, knockout mice and reporter systems will be important for deciphering this complex network of pathogen–host interactions initiating disease.

Cytokines/chemokines amplifying the initial pro-inflammatory signal

During the initial phase of the infection, only very few cells of the intestinal mucosa are in direct contact with the pathogen. Thus, the initial signals emanating from these infected cells are probably not detectable by mucosal gene expression analysis or other methods averaging host responses over the entire tissue. These can only be detected once the signals are amplified and the entire mucosa (or larger domains thereof) has switched to defense. Cytokines and entire response networks induced during this amplification phase were readily detected and include IFN-γ, TNFα, CXCL-2, IL-1β, IL-17, and IL-22 (48, reviewed in 123, 124) (*Fig.* 2). However, due to functional overlaps between these networks and innate immune responses to the pathogen, it has been quite tricky to demonstrate (or refute) the functional importance of individual signal molecules in driving enteropathy or other aspects of the host response. Here, we focus on a few prominent examples.

IFN-γ

IFN-γ was identified early on as a cytokine highly induced in the *S.* Typhimurium-infected mucosa (125) (*Fig.* 5). T-cells and NK cells were identified as prime sources of IFN-γ production. Evidence from knockout mice and cell depletion experiments suggested that this response contributed to full-blown enteropathy at Days 2–4 after infection and that IFN-γ signaling is required for controlling pathogen loads in the mucosal tissue (126–130). These findings are in line with the established role of IFN-γ signaling at the interface between innate immunity (e.g. the enhanced macrophage killing) and adaptive immune responses (e.g. enhanced T-cell responses).

IFN-γ also contributes at very early time points. During the first 8–12 h after infection, IFN-γ-deficient mice and Usp18 mutant animals, which have a defect in IFN-γ production, show a delayed onset of overt mucosal inflammation (131, Hapfelmeier S, Songhet P, Hardt WD, unpublished data). Delayed inflammation and pro-inflammatory gene induction were also observed in $PARP1^{-/-}$ mice, which lack poly (ADP-ribose) polymerase 1 (PARP1), a cofactor of NF-κB-dependent gene expression (9). In particular, interferon-related genes (e.g. *cxcl9*, *igp1*, *cxcl10*, *gbp2*) were expressed with

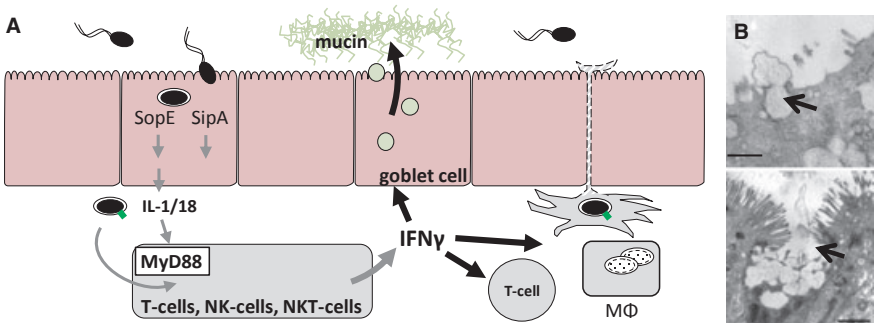


***Fig.* 5. IFNγ coordinates T-cell responses, phagocyte activation, and mucin release by goblet cells.** (A) Working model. (B) Transmission electron micrograph showing pathogen-triggered mucin release into the intestinal lumen of a *S.* Typhimurium-infected C57BL/6 mouse (EM courtesy of Dr. M. Rohde, HZI Braunschweig).

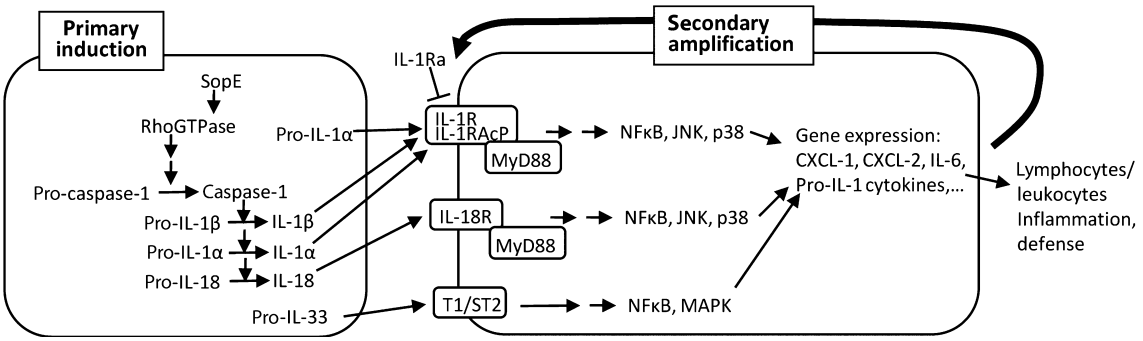


***Fig. 6.* Simplified model of the putative roles of interleukin (IL)-1 family cytokines in the primary induction of inflammation (presumably in enterocytes) and the secondary amplification of the inflammatory response.** Please note that primary induction relies on preformed proteins, while signal amplification hinges on *de novo* gene expression.

delayed kinetics in these mice, in line with a functional importance of the IFN-γ network in this early phase.

A new function of IFN-γ signaling has been discovered recently, i.e. the control of mucin release by the goblet cells of the *S.* Typhimurium-infected mucosa (130). Knockout mice lacking IFN-γR signaling in stromal cells were defective in triggering the release of mucin from large goblet cell vacuoles, a hallmark mucosal response in the streptomycin mouse model (2). Enhanced mucin secretion is thought to limit pathogen access to the epithelial surface and thereby restrict mucosal pathogen invasion (132). Indeed, *muc2*$^{-/-}$ mice, which lack this protective mucin layer covering the gut epithelium, are highly susceptible to sponanteous microbiota-induced colitis and to lethal *C. rodentium* gut infection (133, 134). Thus, IFN-γ seems to coordinate not only T-cell responses and the anti-microbial activity of phagocytes but also goblet cell functions.

Nevertheless, it should be kept in mind that the interpretation of data from knockout mice is complicated by the fact that IFN-γ-deficient mice suffer elevated pathogen tissue loads (and thus MAMP-concentrations). Thus, knockout mice may mount compensatory responses not observed in the wildtype control animals. A detailed understanding of IFN-γ induction, the timing of IFN-γ responses, and cell-type specific effects of IFN-γ induced immunity requires significant future efforts.

Interleukin-1 signaling

IL-1 family cytokines (including IL-1α, IL-1β, IL-18, IL-33) are central inducers of the innate inflammatory immune defense, restricting pathogen growth/survival in the infected tissue. Caspase-1/IL-1-deficiency affects the course of the *S.* Typhimurium infection at least in three ways, i.e. during the initiation of inflammation (via SopE; see above), the amplification of pro-inflammatory signaling within the infected mucosa tissue (*Fig.* 6) and by restricting pathogen spread/-growth within host tissues like the mLN, spleens, and livers (48, 135, 136).

In homeostasis, intestinal epithelial cells (and presumably also *lamina propria* phagocytes) produce just low levels of pro-IL-1α, pro-IL-1β, and IL-18 (48, 137). During the first 10–24 h after orogastric *S.* Typhimurium infection, the mucosa massively upregulates many elements of the IL-1 family network, like IL-1β, IL-33, IL1Ra, IL-1RL1/2/N, IL-18bp, or IL18rap (9, 48, 127, 138) (*Fig.* 6). This induction occurs in epithelial cells and most likely also in phagocytes and/or other cells of the mucosa (48). It seems reasonable to assume that self-amplifying loops are involved. For example, IL-1 cytokines are potent inducers of IFNγ, thus amplifying innate immune responses (e.g. via GM-CSF, TNF-α, IL-1β, CXCL2) and enhancing PMN recruitment and (together with IL-15) augmenting NK cell activity and *Salmonella* colitis (129, 139).

The IL-1 signaling network also includes elements of negative control, presumably to avoid damage by overshooting responses. On the one hand, the expression levels of *casp1*, encoding the key protease required for maturation and release of many IL-1 family cytokines, and *iL-1r1*, encoding the IL-1 receptor, are not affected by mucosal *S.* Typhimurium infection (138). On the other hand, the IL-1 network includes potent inhibitors of IL-1 signaling, e.g. IL-1Ra. This is a soluble protein which binds to the IL-1RI subunit of the IL-1R, thus inhibiting IL-1R signaling. The balance of IL-1Ra and IL-1RI is thought to prevent erroneous induction of inflammation, as indicated by a rare human genetic IL-1R1 deficiency (140) and by IL-1Ra$^{-/-}$ mice, which are prone to spontaneous inflammatory arthritis and arterial inflammation. IL-1Ra is produced both centrally as an acute phase protein by the liver,

delivering high levels of soluble IL-1Ra into the circulation and locally in inflamed tissues, including the *S.* Typhimurium-infected mucosa (138). Thus, IL-1Ra may help limit defense to the site of infection and/or to resolve disease upon pathogen elimination.

In conclusion, IL-1 signaling contributes significantly to initiation and amplification of *S.* Typhimurium inflicted mucosal disease. However, detailed studies are required to decipher how the pro-inflammatory, amplifying, and inhibitory mechanisms coordinate the defense between the different cell types of the intestinal mucosa.

Interleukin-17: no contribution in the first 12 h of the disease?

The IL-17 cytokines IL-17A and IL-17F are strongly induced in the *S.* Typhimurium-infected mucosa, a phenotype apparently requiring Nod1/Nod2 and MyD88 signaling (38, 128, 138, 141) (*Fig.* 2). Based on the importance of IL-17-producing T-cells in innate and adaptive Th17 immune responses (detected by IL-17 production of particular T-cell populations) and initial experiments suggesting an increased susceptibility of IL-17-deficient mice in different bacterial infection models and DSS colitis (142–148), it had been suggested that IL-17 might be a key cytokine amplifying mucosal defense (128, 149). However, this view has been challenged recently. In humans, IL-17 signaling (IL-17RA, IL-17F) is of significant importance for defense against mucocutaneous candidiasis but not against bacterial infection (150). Similarly, in the streptomycin mouse model, antibody-mediated neutralization of IL-17A and IL-17F or IL-17 receptor A deficiency in littermate-controlled experiments did not support a role of IL-17 signaling in the mounting of mucosal inflammation. Moreover, the induction of more than 40 key cytokines and host defense genes typically triggered in response to mucosal *S.* Typhimurium infection were unaffected in the IL-17ra knockout mice (138). Therefore, IL-17 induction should be regarded as a side effect of the acute infection, but it does not seem to affect the pathogen–host interaction during the initiation or the early amplification of the mucosal response.

Currently, the interpretation of most published IL-17 data is quite difficult in respect to the control of bacterial infection. Clearly, this cytokine is expressed early on in response to most bacterial infections. However, the functional importance of the IL-17 response in controlling pathogen burdens (or other 'hard' criteria for a direct function in the pathogen–host interaction) has not been addressed in most reports. Instead, the functional importance of IL-17 induction is often inferred from the early studies on IL-17 signaling-deficient mice. Unfortunately, this early work had suffered from a general lack of littermate controls, an essential requirement for correct interpretation of mucosal infection data. Thus, some of the reported effects on pathogen control might be explained by differences in the genetic background and/or the microbiota affecting the disease kinetics as knockout mice and wildtype controls were obtained from different colonies (discussed below). Most current work on IL-17 responses tends to rely on these early observations thus taking IL-17 induction as a surrogate marker for IL-17-mediated defense. In the streptomycin mouse model for *Salmonella* diarrhea, IL-17 signaling does not seem to affect the initiation or the early amplification phase of *S.* Typhimurium-induced mucosal inflammation, if littermate controls are used (138).

In conclusion, key elements of the amplifying signaling networks have been identified. However, additional work is required for deciphering the sequence of events, for assigning functional roles to particular cytokines and cells, and for deciphering redundancies in this signaling system. Most likely, many of these mechanisms will be of general importance for understanding mucosal homeostasis and pathogen handling by the intestinal mucosa. The streptomycin mouse model is an excellent system for addressing these issues.

Subversion of mucosal inflammation: outcompeting the microbiota by 'ecosystem re-engineering'

In principle, inflammatory responses are employed by the host to clear an invading pathogen. The central importance of this task explains why animals have evolved elaborate sensory mechanisms and inducible defenses to mount such responses. Conversely, pathogens have evolved different ways to inhibit or escape an immune response directed against them. At first, it appeared counter-intuitive that *S.* Typhimurium elaborates a considerable set of virulence factors for triggering (rather than inhibiting) inflammation, a response designed to kill bacteria (see above). Hence the question arises if *S.* Typhimurium might benefit from such pro-inflammatory effector proteins.

Theoretical and experimental studies have shown that pathogens can subvert the host's inflammatory immune response at mucosal surfaces to change growth conditions in a favorable fashion (18, 39, 151–155). In the nasopharynx, *Haemophilus influenzae* can benefit from neutrophil-mediated clearance of *Streptococcus pneumoniae* by opsonophagocytosis. This allows *H. influenzae* to gain a competitive edge and outgrow the competitor in their shared replicative niche (152).

For intestinal pathogens, the situation is even more dramatic. The target organ is already occupied by a dense

and quite stable microbial community, the microbiota, which confers colonization resistance. Intriguingly, colonization resistance can be reverted by mucosal inflammation and S. Typhimurium was shown to rely on triggering gut inflammation to out-compete the microbiota (18). Similar observations were made in the *C. rodentium* model (156). We are just beginning to understand the molecular mechanisms explaining how S. Typhimurium can benefit from the inflammation.

Flagella/chemotaxis and glycoconjugate availability

Flagella were the first S. Typhimurium virulence factor shown to enhance pathogen growth specifically in the inflamed gut (41). This held true for mutants lacking structural flagella components as well as for *cheY* mutants, which can swim but cannot use chemotaxis to follow chemical gradients (Fig. 7). By chemotaxis, the pathogen was shown to move toward the surface of the inflamed mucosa, a rich source of high energy nutrients like mucins, highly glycosylated host proteins released in response to the insult. In line with this observation, the pathogen expressed carbohydrate utilization genes (*mglB*) when approaching the mucosal surface, and chemotaxis was inhibited by oral gavage with galactose. This observation led to the proposal of a 'positive feedback model', whereby S. Typhimurium employs chemotaxis to approach the mucosal surface, not only initiating the inflammatory response [e.g. IFN-γ-mediated mucin excretion (130)] but also for acquiring additional nutrients (glycoconjugates, other nutrients?) fostering further pathogen growth and continued infection: a vicious circle driving prolonged mucosal disease (41) (Fig. 7A,B).

Iron acquisition

Iron is an essential bacterial nutrient, and the restriction of iron availability is a general defense against bacterial infection. Mammalian hosts employ iron-sequestering proteins to limit the availability of this nutrient under homeostasis (lactoferrin, transferrin) and even more so during inflammation. To grow under these conditions, many bacteria have evolved high-efficiency iron uptake systems, like siderophores, which bind iron ions in the environment with very high affinities and cognate ABC transporters allowing harvesting the siderophore-bound iron. In the case of *E. coli*, the siderophore enterochelin facilitates iron import via the *fep* ABC transporter (157). This is thought to allow efficient iron acquisition in the gut of a healthy host. However, in case of inflammation, the host produces and excretes large amounts of the enterochelin-sequestering protein lipocalin-2 into the gut lumen, thus inhibiting enterochelin/*fep*-mediated iron acquisition by *E. coli*. In contrast, S. Typhimurium can bypass this host defense, because it produces salmochelin, a glycosylated variant of enterochelin, which is not bound by lipocalin-2, and a dedicated ABC transporter (*iro*) (149) (Fig. 7C). Through this mechanism, S. Typhimurium can still acquire iron in the inflamed gut, explaining how the pathogen can profit from triggering mucosal inflammation in order to out-compete commensals like *E. coli*.

Anaerobic respiration

It was discovered recently that the PMNs, which transmigrate into the gut lumen as part of the inflammatory defense, are also subverted by S. Typhimurium (Fig. 7C). In particular, they con-

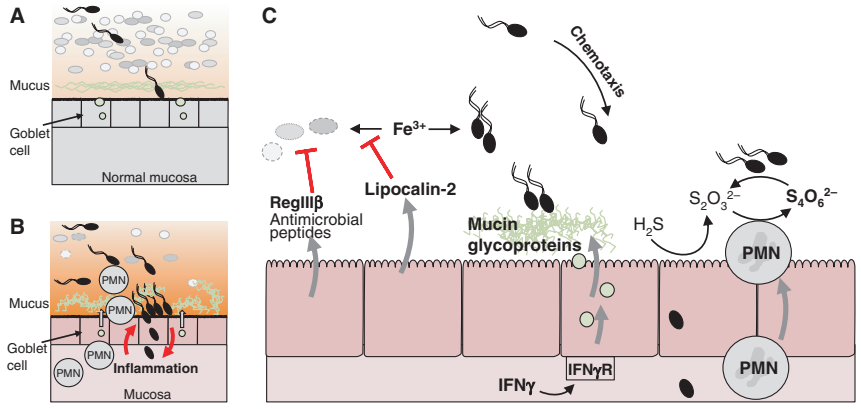


Fig. 7. S. Typhimurium can subvert the host's inflammatory response to outcompete the microbiota. (A) To initiate colonization, S. Typhimurium employs chemotaxis to accumulate at the mucosal surface. (B) Vicious cycle emerging as the pathogen capitalizes on the altered environmental parameters present in the inflamed gut. (C) Mechanisms contributing to the growth advantage of the pathogen in the inflamed intestine. Gray, microbiota; black, S. Typhimurium.

vert thiosulfate, a detoxification product of H_2S (produced by some members of the microbiota), into tetrathionate (158). *S.* Typhimurium encodes the ttr operon, allowing the pathogen to use tetrathionate as a terminal electron acceptor for anaerobic respiration, thus converting tetrathionate back to thiosulfate. The regeneration of tetrathionate by the NADPHoxidase-mediated oxidative burst activity of the PMN guarantees a constant supply of this terminal electron acceptor, as long as PMNs are present in the gut lumen (158). The anaerobic tetrathionate respiration does not generate as much ATP as the aerobic respiration. However, it generates significantly more ATP than fermentation, which is used by most commensals in the gut. This explains why *S.* Typhimurium can use sugar substrates (mucins, see above) more efficiently and thereby out-grow the competing microbiota in the inflamed gut.

Anti-microbial peptides, RegIIIβ

Under homeostasis, anti-microbial peptides are of key importance for preventing microbiota-access to the stem cell zones of the crypts (159). In response to pathogen insult, the mucosa dramatically increases the production and release of such anti-microbial peptides, including the C-type lectins RegIIIβ and RegIIIγ, which display distinct spectra of anti-microbial activity (160–163). For example, RegIIIβ kills *C. butyricum*, *Lactobacillus reuteri*, and different *E. coli* strains but not *E. faecalis*, *L. murinus*, and *S.* Typhimurium. The mucosal *S.* Typhimurium infection triggers massive production of RegIIIβ by enterocytes (163). In the intestine, the anti-microbial activity spectrum of RegIIIβ provides *S.* Typhimurium with a competitive edge over *E. coli* strains of the normal microbiota (*Fig. 7C*). Thus, the resistance to the host's anti-microbial peptide response seems to represent another mechanism whereby the pathogen benefits from eliciting mucosal inflammation.

These findings provide first evidence how *S.* Typhimurium can subvert the inflammatory host response for its own benefit. We expect that many additional mechanisms will be discovered which contribute to this 'ecosystem re-engineering' strategy. It seems reasonable to predict that the underlying mechanisms may be of general importance for other enteropathogenic bacteria. They all face similar challenges, as the niche to be colonized is already occupied by a very dense microbial community, the microbiota.

Streptomycin-treated mice, a model for studying adaptive mucosal immune responses

The streptomycin mouse model has been extended to study recovery from the primary infection and the generation of adaptive mucosal immunity (24). In immune-competent humans, *S.* Typhimurium and other non-typhoidal *Salmonella* (NTS) strains generally cause a self-limiting enterocolitis. Diarrheal symptoms cease after several days, and the patients mount a protective, cellular, and humoral immune response (i.e. mucosal *Salmonella*-specific secretory sIgA). Nevertheless, the pathogen can be detected in the asymptomatic patient's stool for weeks and even months, which can lead to environmental spread of the infection (164–166). Antibiotic therapy is usually not indicated, as it can prolong pathogen excretion and increase the risk of clinical relapse due to collateral damage to the endogenous microbiota (167).

We recently modified the streptomycin mouse model to analyze the recovery phase from an acute *S.* Typhimurium infection (24) (*Fig. 8A*). Streptomycin-treated C57BL/6 mice were infected with the attenuated *S.* Typhimurium mutant strain *sseD*. This strain causes colitis after 6–24 h postinfection, spreads to the MLNs, but does not cause a life-threatening systemic infection (37). In line with earlier work (33), the intestinal pathology was resolved after 5–21 days postinfection (24). Within 40 days postinfection, wildtype C57BL/6 mice fully recovered from intestinal disease and developed protective immunity against a secondary 'challenge infection'. In parallel, fecal pathogen shedding declined in many animals. However, a significant number of animals displayed prolonged pathogen shedding at high levels. These observations suggested that the resolution of inflammation, the generation of adaptive immunity, and pathogen clearance from the stool are not strictly interdependent and can be studied separately from each other. In the following section we discuss the role of (i) the adaptive immune system; (ii) the mucosal IgA response; and (iii) the gut microbiota in recovery from an acute NTS infection.

Role of the adaptive immune system in resolving the enteric *S.* Typhimurium infection

The adaptive immune response to oral *S.* Typhimurium infection has been extensively studied in the last two decades (reviewed in 168–171). In the majority of studies, the typhoid model (mice orally immunized with *S.* Typhimurium without previous antibiotic treatment) was used. The course of infection and symptoms in this model resembles human typhoid fever caused by *S. enterica* serovar Typhi: bacteria invade the mucosa via M-cells, colonize Peyer's patches of the small intestine, and disseminate to systemic sites, cause bacteremia and subsequently colonize MLNs, spleen, and liver. However, no enteric symptoms such as diarrhea or inflamma-

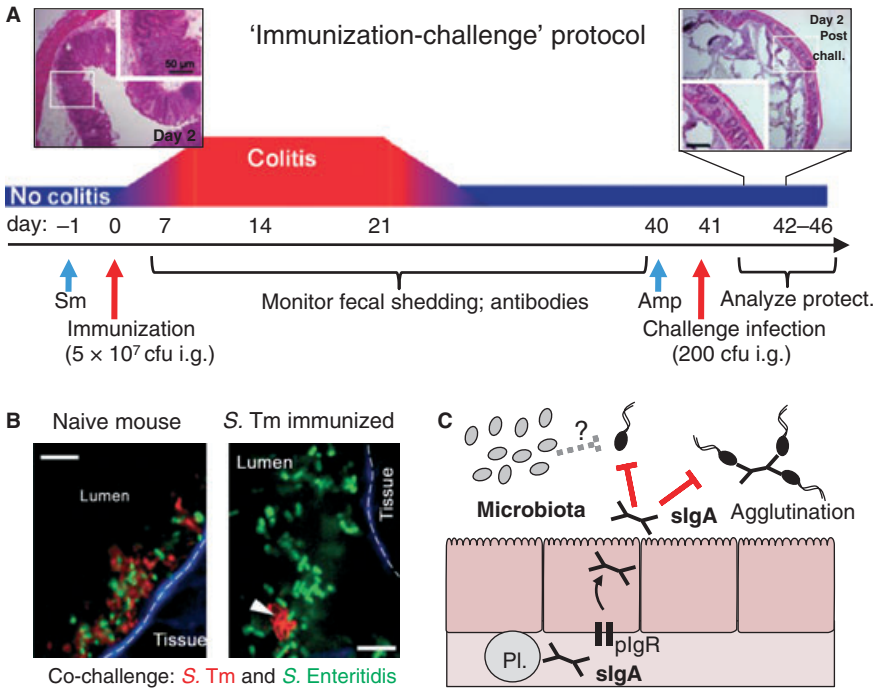


***Fig. 8.* Modified streptomycin mouse model for studying later phases of the infection.** (A) Immunization-challenge protocol. Reproduced with permission from Endt *et al.* (24). (B) O-sidechain-specific sIgA protects from mucosal disease by pathogen aggregation in the gut lumen and prohibiting pathogen access to the epithelial surface. Reproduced with permission from Endt *et al.* (24). (C) Protective effects of the microbiota and the sIgA response.

tion are observed. This is due to colonization resistance mediated by the resident intestinal microbiota which interferes with intestinal pathogen colonization (13). In the typhoid fever model, mice orally immunized with an attenuated *Salmonella* vaccine strain overcome the initial phase of infection, eliminate S. Typhimurium from systemic sites, and develop adaptive immunity.

S. Typhimurium induces systemic expression of a panel of pro-inflammatory cytokines as TNFα, IFNγ, IL-1, IL-6, IL-12, and IL-18 (reviewed in 168). This leads to recruitment and activation of phagocytes and effective containment of the pathogen. Consequently, a macrophage activating Th1-type immune response is crucial for survival of a S. Typhimurium infection: mice lacking $CD4^+$ T-cells (nude, $TCR\beta^{-/-}$, $CD4^{-/-}$, $CD28^{-/-}$, MHC class II) fail to resolve the primary infection (i.e. clear the pathogen from systemic sites). $CD8^+$ T-cells are also involved in generation of immunity but seem to be of lower importance (172–174). In contrast, mice lacking functional B cells can clear *Salmonella* from the reticuloendothelial system but fail to generate a protective immune response against challenge infection. This has mainly been attributed to the lack of antibodies but the exact mechanism of antibody-mediated protection has remained unclear (175) (discussed below). In conclusion, effective immunity against *Salmonella* infection in the typhoid fever model requires the combined action of a Th1-response and antibodies.

The modified streptomycin mouse model allows the analysis of the immune responses after enteric salmonellosis, involving concomitant intestinal pathology, and thus closely resembles human NTS infection. Streptomycin-treated $TCR\beta^{-/-}\delta^{-/-}$ and $JH^{-/-}$ mice, lacking T and B cells, respectively, harbored similar pathogen loads in the MLNs and spleen as wildtype controls at day 40 postinfection (24) and they were able to resolve gut inflammation.

What are the mechanisms underlying the resolution of *Salmonella*-induced colitis? In the typhoid fever model, $CD4^+$ $Foxp3^+$ Treg cells are actively involved in suppressing anti-*Salmonella* effector $CD4^+$ T-cell activation in the first 3 weeks after infection in the spleen (174). It is yet unclear which role Treg cells play in *Salmonella* gut tissue infection. Interestingly, large numbers of $CD4^+$ infiltrates were evident in the cecal *lamina propria* of convalescent mice (24), yet their cytokine profile, subtype, or functionality is unknown. Milestone work in mucosal immunology has recently revealed that pro- and anti-inflammatory T-cell subsets are modulated by the commensal microbiota. Commensal *Clostridium* spp. as well as *Bacteroides fragilis* and the altered Schaedler flora (ASF) induced $CD4^+$ $Foxp3^+$ Treg cells (176–178). In contrast, segmented filamentous bacteria

(SFB) are known for inducing predominantly pro-inflammatory Th17 responses (179). Thus, it is conceivable that the microbiota composition could directly or indirectly affect susceptibility to and resolution kinetics of *Salmonella*-induced colitis. Future work will have to address this issue.

Induction of a protective, *Salmonella*-specific sIgA response

Oral S. Typhimurium infection in the typhoid model leads to a potent antibody response. The major target is bacterial lipopolysaccharide (LPS), but proteinaceous antigens such as flagellin have also been reported (24, 180, 181). Ever since, it has been a matter of debate how antibodies could mediate immunity against an intracellular pathogen. In the challenge infection, immunoglobulins could block S. Typhimurium at different stages: secretory IgA and IgM can bind pathogens in the gut lumen. However, this was not confirmed using $pIgR^{-/-}$ mice (182). Furthermore, IgM and IgG could enhance phagocyte uptake in the Peyer's patches, lead to complement-dependent pathogen killing, or facilitate lysosomal pathogen degradation within phagocytes (183). Finally, antibodies may impede S. Typhimurium cell-to-cell spread.

In the streptomycin model, convalescent mice mount a strong S. Typhimurium-specific antibody response. IgM and IgG directed against bacterial proteins and LPS is detectable in the serum of immunized mice in the first week after infection. After 2 weeks, secretory (s)IgA with the same specificity is secreted into the intestinal lumen. Moreover, sIgA directed against the LPS O-sidechain of S. Typhimurium was absolutely required for protective immunity against colitis in challenge infections with wildtype S. Typhimurium (24). In line with this, mice lacking B cells ($JH^{-/-}$), IgA ($IgA^{-/-}$) (184), or the poly-Ig receptor ($pIgR^{-/-}$) (185), which are necessary for translocation of sIgA produced by *lamina propria* plasma B cells across the epithelial barrier, were not protected from enteropathy (24).

sIgA was found to protect by binding the pathogen already in the gut lumen, i.e. by agglutinating the pathogen, by reducing its overall growth rate in the gut lumen and by limiting access to the epithelial surface in a O-sidechain-specific manner (24) (*Fig.* 8B,C). The protection was highly specific, as vaccination with S. Typhimurium *sseD* only protected against challenge with wildtype S. Typhimurium, but not against challenge with an isogenic, O-sidechain deficient mutant (*wbaP*) (186) or challenge with a closely related serovar Enteritidis strain which expresses a non-cross-reactive O-antigen (24). Most likely, the high protective efficiency of the anti-O-sidechain antibodies is attributable to the highly polymeric and surface-exposed nature of the *Salmonella* O-antigen, enabling strong agglutination by sIgA.

How is this protective sIgA response to enteric S. Typhimurium infection generated? Antibodies against highly polymeric bacterial antigens can be induced in a T-cell-independent fashion by B1 cells (187). Class-switch recombination of B1-cells occurs independent of CD40 independently in the *lamina propria* in the presence of an appropriate cytokine environment (IL-10, TGF-β). The process is mediated by the interaction of B cells and *lamina propria* dendritic cells via B-cell activating factor and a proliferation-inducing ligand (188). However, such natural antibodies generally have a low antigen affinity. Nevertheless, natural antibodies can have some protective effect against oral *Salmonella* infection in the typhoid fever model (189). Conventional B2 cells require T-cell help and germinal center formation for affinity maturation and IgA isotype switching (190–192).

In the streptomycin model, $TCR\beta^{-/-}\delta^{-/-}$ mice did not mount a protective LPS-specific sIgA response, suggesting that protection relies on a B2-type T-cell dependent mechanism (24). So far, there is only little experimental data on T-cell-dependent generation of antibodies directed against bacterial polysaccharides. Nevertheless, the underlying mechanism is of significant interest. Unlike peptide antigens, polysaccharides cannot be processed and presented by MHC class II molecules to T-cells (193). Instead, they might directly bind to MHC class II on antigen-presenting cells or bind to a cognate TCRs and lead to $CD4^+$ activation.

Furthermore, the route of primary S. Typhimurium infection seems to affect the immunization efficiency, as systemic immunization (intraperitoneally) did not yield protective mucosal sIgA. Immunization with a non-invasive S. Typhimurium mutant also failed to generate protective immunity (Δ*invGsseD*) (24). This might resemble earlier findings in the typhoid fever model, which demonstrated that the route of S. Typhimurium Peyer's patch entry (via TTSS-1 or via DC-sampling) affects the pathogen-specific IgA response (194). Based on these observations, the poor vaccination efficiency of the non-invasive mutant Δ*invGsseD* in the streptomycin model might be attributable to an unfavorable entry route into the tissue. Alternatively, the fast displacement of this mutant from the gut lumen in the absence of inflammation (i.e. via the competing microbiota) might simply reduce the duration or intensity of pathogen exposure below the level required for mounting an efficient response. Clearly, the wealth of available tools make streptomycin-treated mice a highly promising tool for studying adaptive mucosal sIgA responses against bacteria.

The microbiota mediates pathogen clearance

Strikingly, sIgA was dispensable for pathogen clearance from the gut lumen. $IgA^{-/-}$ mice cleared *S.* Typhimurium at similar rates as wildtype littermate controls (WT and $IgA^{+/-}$) (24). Instead, competitive displacement by the microbiota was essential for terminating pathogen shedding by the convalescent mice.

Streptomycin treatment transiently depletes the microbiota by 90–99% and abolishes colonization resistance (2, 18–20, 29, 30). Five days after streptomycin treatment, density and phylum-level composition is restored and the microbiota starts overgrowing the pathogen in the absence of inflammation (18). In contrast, in the inflamed intestine, the microbiota is incapable of re-growing and outcompeting the pathogen (18, 156, 195) (discussed above).

In the vaccination model (*Fig.* 8), it takes the mice approximately 5–21 days to resolve gut inflammation. Afterwards, one would expect that the microbiota should again out-compete the pathogen in the intestinal lumen, thus terminating pathogen shedding. Indeed, this happened in many mice. However, in approximately one-third of the animals, high levels of pathogen excretion persisted for up to 80 days (24). This 'asymptomatic excretor' state is similar to the situation in human *Salmonella*-infected patients: asymptomatic excretors show no clinical signs of inflammation (intestinal pathology) but still shed high levels (10^5–10^9 cfu/g) of the pathogen into the environment for up to 40 days postinfection. The asymptomatic excretor state that we observed in our model was not due to a failure of the mice to generate a pathogen-specific sIgA response (24). We hypothesize that in asymptomatic excretors, parts of the microbiota are rendered non-functional or lost. This may occur either during antibiotic treatment or via pathogen-triggered inflammation. In fact, antibiotic therapy can result in long-term microbiota changes and selective loss of species (i.e. in the case of ciprofloxacin-treatment, which is standard therapy of complicated human NTS infections), and/or alter mucosal immune homeostasis (27, 196, 197). Thus, antibiotic-inflicted alterations/losses of key members of the microbiota might compromise pathogen elimination in long-term asymptomatic excretors. In an experimental setting, introduction of an unmanipulated donor animal could indeed rescue asymptomatic excretors via 'fecal transplantation' (24). Again, the streptomycin-treated mice offer an excellent model for studying the underlying mechanisms.

Gnotobiotic mice colonized with a low-complexity type of microbiota (LCM) have already emerged as another powerful variant of the streptomycin mouse model for studying host factors and the function of the microbiota in pathogen clearance (24). LCM mice lack colonization resistance and are susceptible to enteric salmonellosis without the need of antibiotic treatment (12, reviewed in 13). If immunized with the attenuated *S.* Typhimurium strain *sseD*, LCM mice initially develop colitis and recover from mucosal disease in a similar way as streptomycin-treated conventional mice. They also mount a protective sIgA response by 40 days after infection. However, despite the presence of high-affinity anti-LPS sIgA, 100% of the animals keep shedding high loads of *S.* Typhimurium for at least 80 days (24). The shedding could only be terminated by fecal transplantation, i.e. by contact with a normal mouse shedding a complex, healthy microbiota, which was shown to be transferred to the infected LCF mice and which outcompeted the pathogen in the gut lumen. So far, both, the components of this complex bacterial cocktail important for mediating clearance as well as the underlying mechanisms have remained unknown.

In conclusion, work on the streptomycin model reveals that microbiota integrity might be irreversibly impaired by *Salmonella*-induced intestinal inflammation and/or antibiotic therapy and induce asymptomatic fecal shedding and transmission of the pathogen. This may support the notion that fecal transplants might offer a cure of such bacterial infections (198). The streptomycin mouse model and its variant using LCF mice offer excellent experimental tools for analyzing the microbial species and the mechanisms driving pathogen clearance after the end of an acute infection.

Microbiota: key players in each stage of the mucosal *Salmonella* infection

The microbiota plays a central role at each stage of the *Salmonella* diarrhea infection cycle and each function can be analyzed in the streptomycin mouse model (*Table* 2). Upon ingestion by a naive host, the microbiota efficiently blocks pathogen growth by colonization resistance (2, 13). The mechanisms underlying colonization resistance as well as the parts of the gut microbiota central for the generation of this

***Table* 2. Complementary protection from mucosal infection by the microbiota and by sIgA**

Stage	Function	Microbiota	sIgA
1st infection: colonization	CR	++++	(+)*
1st infection: acute disease	CR?	-	(+)*
1st infection: convalescence	Pathogen elimination	++++	+
Re-infection	As above	As above	++++

*Innate sIgA may contribute slightly; ++++strong protection; +weak protection; -no protection.

condition are poorly understood. Direct and indirect mechanisms are conceivable. The microbiota can directly inhibit pathogens by producing toxic metabolites such as the short-chain fatty acids acetate and butyrate, which are known to repress *Salmonella* virulence gene expression (199, 200). At the same time, acetate produced by *Bifidobacterium* spp. strengthens intestinal barrier function and protects mice against lethal challenge with Shiga-toxin producing *E. coli*. One study has recently compared the efficacy of fructose metabolism between different *Bifidobacterium* spp. strains, showing how this may vary depending on the microbial community structure. Only those strains harboring a certain ABC-type transporter were able to produce high levels of acetate and protect mice from lethal challenge infections (201).

Microbiota complexity generally positively correlates with colonization resistance against oral *S.* Typhimurium infection (13, 25). Thus, it may also be conceivable that not a specific type or group of commensals but rather different combinations of gut microbes can confer colonization resistance. In an evolutionary context, this would make sense. There is high variability in microbiota composition in the form of different 'enterotypes' in the human population (1). It would be interesting to see if all enterotypes provide the same degree of colonization resistance or if certain enterotypes may confer 'enhanced colonization resistance' against particular pathogens. Epidemiological studies and experiments in gnotobiotic mice associated with different human enterotypes would yield important information.

Different enterotypes have also been described in mice. C57BL/6 mice obtained from different suppliers do not necessarily harbor the same microbiota. Different microbiota communities can lead to altered mucosal immune homeostasis (IL-17 production by $CD4^+$ T-cells) or affect susceptibility to oral *S.* Typhimurium infection (12, 148). As discussed in detail below, this is of significant practical importance for the design of knockout mouse experiments functionally analyzing the host's response to mucosal infection. Thus, gnotobiotic mice are an extremely valuable tool for the analysis of the mechanisms underlying microbiota–pathogen interactions. They offer an excellent model system to experimentally assess the contribution of specific strains and even enterotypes to disease susceptibility in the future.

In immunocompetent humans and in the *S.* Typhimurium vaccination model alike, inflammation ceases after a few days to several weeks (24). The mechanisms driving remission have not been analyzed and will be an interesting topic for future work. Once the inflammation has ceased, the microbiota can re-establish and outcompete the pathogen in the gut lumen, a key event for termination of pathogen shedding (*Table* 2). Again, the molecular basis remains to be established.

Upon pathogen clearance, the microbiota seems to regain its preinfection state, thus re-establishing colonization resistance against infection with the same or with other pathogens (24). This intriguing property of the intestinal microbiota to recover after major insults inflicted to the gut ecosystem is also termed 'resilience' (202). It may represent an ancient mechanism which has protected the host's intestine from infection since the early stages of animal evolution. The mechanisms driving resilience and microbiota stability over time are unknown. The LCM-model infection data and the successful treatment of asymptomatic excretors by fecal microbiota transfer suggest that contamination with 'healthy' microbiota might be a key mechanism. In any case, the microbiota and the sIgA seem to act in a complementary fashion protecting the host from disease during the initial encounter and subsequent infections with the same pathogen (*Table* 2).

Effects of the mouse genetic background on systemic and mucosal infection

General importance of Slc11α1 in murine *Salmonella* infections: typhoid fever model

The susceptibility to lethal systemic infection by intracellular parasites such as *Salmonella* spp. is in many cases linked to a mutation in the solute carrier family 11a member 1 locus (Slc11α1; formerly Nramp-1 for natural resistance macrophage-associated protein) (203, 204). Slc11α1 is located on the mouse chromosome 1 and encodes a membrane-associated divalent cation anti-porter found in endosomes and lysosomes of macrophages in spleen and liver (205–207), and in a subset of dendritic cells in the *lamina propria* of small and large intestines (208). Susceptible mouse strains (e.g. C57BL/6J and BALB/cJ) encode a G → A transition resulting in a Gly to Asp substitution at position 169 of the Slc11α1 protein (209, 210). This mutation, in the second hydrophobic helical domain of Slc11α1, is thought to impair membrane association and function of the protein (209).

In the typhoid fever model, it is well established that *slc11α1*$^{-/-}$ mouse lines are permissive for systemic *S.* Typhimurium growth, while *slc11α1*$^{+/+}$ mouse lines can restrict systemic disease (35, 203, 211, 212). Several mechanisms were proposed to explain how Slc11α1 restricts the growth of pathogens within the host's phagocytes (213, 214). First, Slc11α1 may affect the Fe^{2+} concentration within the phagosome, though the direction of divalent metal ion transport is not entirely clear. Changing [Fe^{2+}] in either way could restrict pathogen growth: on the one hand, iron is the catalyst of the

Haber-Weiss/Fenton reaction that generates highly bactericidal hydroxyl radicals; on the other hand, Fe^{2+}, Mn^{2+}, and Zn^{2+}starvation impairs bacterial growth and their resistance against reactive oxygen species (215). Moreover, iron starvation was shown to enhance the expression of the TTSS-2 genes (216). Second, Slc11α1 could play a role in the maturation of the SCV (217). The lack of Slc11α1 in RAW-cells and in resident peritoneal macrophages obtained from *slc11α1*$^{-/-}$ mice (i.e. Balb/c or C57BL/6 background) impairs the acquisition of the mannose-6-phosphate receptor by the vacuoles, modifies the delivery of lysosomal enzymes to the SCV and affects the killing of the bacteria. Third, Slc11α1 may accelerate the inflammatory response during *S.* Typhimurium infection (208). Valdez and co-workers suggested that Slc11α1-expression by particular $CD11c^{+}CD103^{-}$ DCs of the small intestinal mucosa can enhance the production of pro-inflammatory cytokines like IL-6, IL-12, and TNF-α after oral *Salmonella* infections. Future work will have to resolve the relevant mechanism.

Supershedder model in Slc11α1$^{+/+}$ mice

In conventional, Slc11α1$^{+/+}$ 129SvEv mice, which harbor a normal microbiota, the orogastric infection with *S.* Typhimurium leads to a chronic systemic infection (218). The pathogen is present at significant densities in livers, spleens, gall bladders, and particularly in the mLN for 180–365 days. Lethal disease is controlled by IFNγ-dependent defense. During this chronic infection, follow up work detected the occasional appearance of 'supershedders', i.e. mice displaying severe mucosal inflammation, pathogen loads of up to 10^{10} cfu/g in the intestine, and high levels of fecal shedding (219). These phenotypes suggest that the transition of nonshedders to supershedders might be driven by the very same mechanisms as observed in the streptomycin model (*Figs* 2 and 7). The supershedder model should be of significant interest for studying how the microbiota, the host, and/or the pathogen may affect the frequency of the transition. However, mechanistic studies are complicated by the unpredictable nature of the onset of supershedding.

S. Typhimurium colitis in streptomycin-treated Slc11α1$^{+/+}$ mice: from acute to chronic mucosal inflammation

Based on the key role of Slc11α1 in the *Salmonella*-macrophage interaction and the importance of this interaction in eliciting mucosal inflammation (*Fig.* 3), it was of significant interest to analyze *Salmonella*-inflicted mucosal disease in Slc11α1$^{+/+}$ animals. Interestingly, streptomycin-treated Slc11α1$^{+/+}$ mice (129SvEv, DBA/2) developed an acute inflammatory response with approximately the same kinetics and pathology as the Slc11α1-negative mouse lines at days 1–3 postinfection (Balb/c, C57BL/6) (35, 130). We assume that slight differences in the kinetics of the onset of enteropathy, as observed in another study (214), might be attributable to differences in the microbiota and/or microbiota-induced differences in the mucosal defense status of the mouse colonies employed (discussed below). Overall, the initial phase and amplification mechanisms seem to be equivalent in *slc11α1*$^{+/+}$ and *slc11α1*$^{-/-}$ mice, arguing that Slc11α1-dependent restriction of pathogen growth in mucosal phagocytes may have only minor effects at this stage of the disease. The reasons for this (e.g. lack of mucosal Slc11α1-expression; Slc11α1-independent growth in some mucosal cell types) will be an interesting topic for future work.

After day 3 postinfection, the disease progression differs significantly between Slc11α1$^{+/+}$ and Slc11α1$^{-/-}$ mice. By day 5–6 postinfection, *S.* Typhimurium reaches extremely high systemic tissue loads in the Slc11α1$^{-/-}$ mice, and the animals become moribund. Thus, Slc11α1$^{-/-}$ mice cannot be used for studying the enteric disease inflicted by wildtype *S.* Typhimurium beyond day 4 postinfection. In contrast, Slc11α1$^{+/+}$ mice can control the lethal pathogen growth at systemic sites sufficiently well to allow long-term disease studies (35). High pathogen loads in the gut lumen and the feces (10^{7}–10^{8} bacteria/g) as well as at systemic sites (up to 10^{4} bacteria in mesenteric lymph nodes and liver) remain for months. Within 10–20 days, these animals develop typical signs of chronic gut infection, including not only crypt destruction, ulceration, crypt abscesses, but also overshooting regeneration, crypt branching, fibrosis and inflammation of the gall duct epithelium (cholangitis) (35, 212) (*Fig.* 9). This resembles the pathology of inflammatory bowel disease patients and might therefore represent a suitable model for mechanistic studies. Moreover, as 100% of the animals do develop cholangitis by day 14 postinfection, this infection model should be well suited for studying microbe-induced gall duct cancer. Cholangitis is observed in 2–4% of all patients suffering from chronic colitis and may represent a risk factor for colangio-colorectal carcinoma. In fact, patients chronically infected with *S.* Typhi have a significantly increased risk of developing this type of cancer (220–225). This explains the interest in the Slc11α1$^{+/+}$-variant of the streptomycin mouse model as a robust experimental system for analyzing chronic infection of the gastrointestinal tract in order to decipher molecular mechanisms linking long-term colitis and cancer.

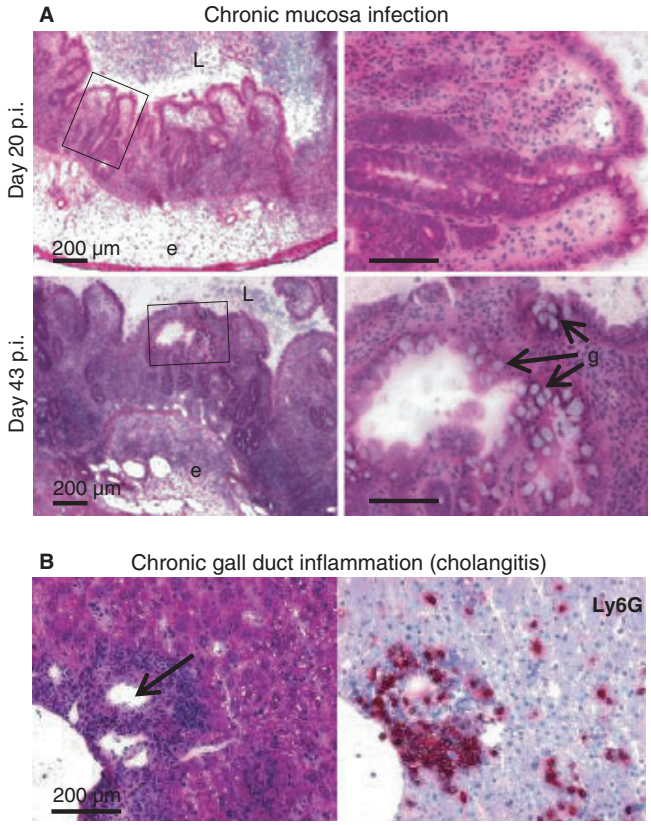


***Fig. 9.* Chronic mucosa and gall duct inflammation in *slc11α1*$^{+/+}$ mice.** Animals were chronically infected with wt *S.* Typhimurium. The images are reproduced with permission from Stecher *et al.* (35). L, gut lumen; e, edema in submucosa; g, goblet cell.

The major drawback of the Slc11α1$^{+/+}$-variant of the streptomycin mouse model is that most of the available transgenic and knockout mouse lines are maintained in the Slc11α1$^{-/-}$ C57BL/6 genetic background. Thus, the use of *S.* Typhimurium mutants with reduced systemic virulence represents an attractive alternative. Mutants in *aroA* (31, 212) and *sseD* or *ssaV* have already been used (non-functional TTSS-2) (24). Both types of *S.* Typhimurium mutant induce acute colitis (via the classical pathway) (*Fig.* 3) in the streptomycin mouse model (in *slc11α*$^{-/-}$ and $^{+/+}$ mice) which is almost as strong as that triggered by wildtype *S.* Typhimurium in the early stages of infection (24, 31, 33, 212). Thus, attenuated *S.* Typhimurium strains may allow studying the role of host genes of choice in chronic gut infection, even if the respective allele is only available in a Slc11α1$^{-/-}$ genetic mouse background.

All other things being equal? The impact of the microbiota

Regarding the composition of their microbiota, even isogenic mice from different breedings do differ (12). As described in the preceding chapter, the microbiota plays a major role during *S.* Typhimurium infection by modulating the host immune response, impairing pathogen colonization, and accelerating the clearance of the pathogen. Thus, it is not surprising that the microbiota composition can affect the disease in the *S.* Typhimurium diarrhea model.

Microbiota affects colonization resistance

The degree of colonization resistance against orogastric *S.* Typhimurium infection is strongly affected by the host's microbiota composition. Antibiotic-treated mice lacking an intact microbiota and germ free mice do not show colonization resistance (2, 19, 20, 219, 226, 227). Similarly, mice harboring a defined low-complexity gut flora (4–20 species; LCM mice) do not display colonization resistance (12, 24). In these animals, colonization resistance could be re-established by co-housing with conventional mice (specified pathogen free mice), which facilitated the acquisition of a conventional microbiota. Moreover, we have observed differences in the degree of colonization resistance between C57BL/6 mice originating from different conventional mouse colonies. Here, certain members of the normal microbiota could be identified as colonization resistance 'indicators' (12) (*Fig. 10A*). High *L. reuteri* stool loads correlated with pronounced colonization resistance against *S.* Typhimurium gut colonization and enteropathy [without prior antibiotic treatment (12)]. Conversely, high *E. coli* stool loads correlated with low-level colonization resistance (*Fig. 10A*). In mice displaying high loads of commensal *E. coli*, orogastric *Salmonella* infection led to efficient pathogen growth in the gut lumen and colitis without a need for prior antibiotic treatment. The underlying mechanism is still unclear. On the one hand, the indicator species might directly inhibit or foster *Salmonella* growth. On the other hand, it seems more likely that the indicator species are indicative of a favorable (or unfavorable, *L. reuteri*) ecological niche for the colonization by a phylogenetically close relative. This would be in line with observations in other ecosystems (228).

Microbiota can affect the degree of *S.* Typhimurium inflicted enteropathy

The microbiota composition does not seem to affect the disease parameters of the streptomycin mouse model for *Salmonella* diarrhea in most cases. This is indicated by the fact that numerous laboratories worldwide, using mice from various sources, have been able to establish the disease model successfully in their animal facilities. Thus, the basic model is quite robust.

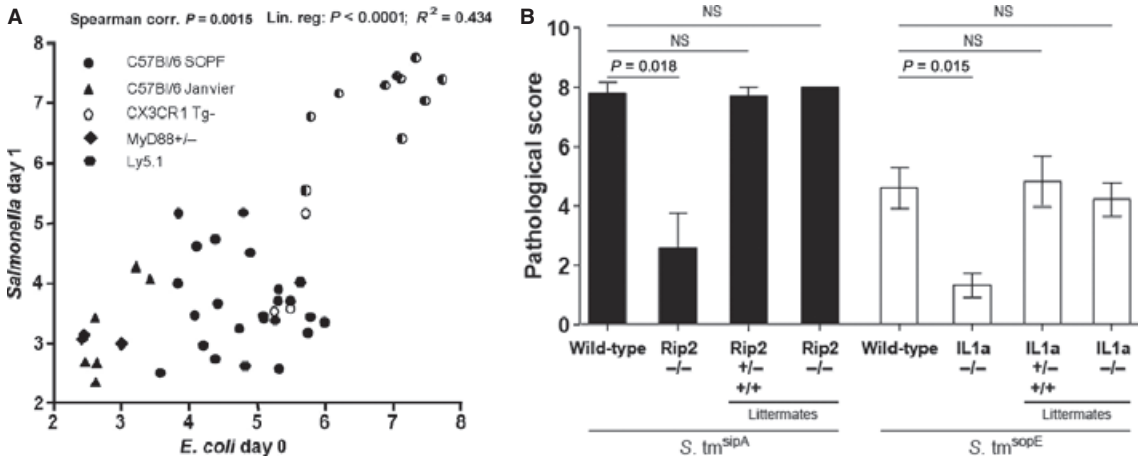


***Fig. 10.* Microbiota composition can afffect the S. Typhimurium infection.** (A) The composition of the microbiota correlates with the degree of colonization resistance. Normal unmanipulated conventional mice (6–12 weeks; symbols indicate different sources) were infected with 5.10^7 cfu of *S.* Enteritidis wildtype by gavage. Fecal *E. coli* titers before infection were determined (*x*-axis; log10 cfu/g). One day postinfection, *S.* Enteritidis titers in the feces were determined (*y*-axis; log10 cfu/g). Half-filled symbols indicate animals with overt mucosa inflammation. Reproduced with permission from Stecher *et al.* (12). (B) Importance of littermate controls. Mean pathological scores of streptomycin pretreated C57Bl/6 wildtype mice (ETH Zürich colony), *rip2*$^{-/-}$, *Il1a*$^{-/-}$ (from other colony), and inbred littermates mice (i.e. *rip2* or *Il1a* −/−, +/− or +/+; bred at ETH Zürich) 48 h after oral infection with 5.10^7 cfu of *S.* Tm^{sipA} (M716 *sseD*::*aphT* Δ*sopB* Δ*sopE* *sopE2*::*pM218*) or *S.* Tm^{sopE} (M717 *sseD*::*aphT* Δ*sopB* *sopE2*::*pM218* Δ*sipA*). Significance of the mean score variations between groups were calculated (Mann–Whitney *U*-test. NS, non-significant, $P > 0.05$). Error bars represent the standard error of the mean.

When using knockout mice to identify signaling pathways which may contribute to enteropathy, however, we have noticed that the microbiota might affect disease kinetics and/or the degree of enteropathy. Typically, such knockout mice are received from commercial suppliers or collaborating laboratories. It seems straightforward to compare the disease parameters to isogenic wildtype mice from the same supplier (where they are bred in a separate colony!) or another source. In our laboratory, we have performed this type of approach to assess the role of *rip2* and *IL1a* in SipA- or SopE-dependent colitis. Rip2 is a kinase involved in the signaling downstream of Nod1 and Nod2, constituting an important arm of the innate immune response (229) (*Fig. 4A*). IL1α is a pro-inflammatory cytokine and an important effector cytokine released upon inflammasome/caspase-1 activation (230) (*Fig. 4 and 6*). Indeed, these initial experiments suggested a significant role of *rip2* and *IL1a* in driving SipA- or SopE-dependent colitis (S.Tm^{sipA} or S.Tm^{sopE}) (48) (*Fig. 10B*). To verify these observations, we set up +/− × +/− breedings and compared disease parameters between the +/+ and the −/− littermates. This strategy should alleviate most differences in microbiota composition (and genetic makeup) (231). Strikingly, in these experiments, the *rip2*$^{-/-}$ and *IL1a*$^{-/-}$ mice displayed equivalent enteropathy as the wildtype littermate controls. Thus, *rip2* and *IL1a* are not essential for SipA- or SopE-dependent colitis. Clearly, littermate controls are of key importance to avoid misinterpretation and may help to resolve seemingly contradictory findings (232, 233) (*Fig. 10B*). This notion might be of general importance for gut mucosa immunology, as indicated by the profound effect of the microbiota composition on the susceptibility to DSS colitis (234) and numerous other publications demonstrating a key role of the microbiota in the maturation and responsiveness of the mucosal immune system (179). This strongly suggests that the gut flora composition could constitute a confounding variable that must be taken into account when the phenotype of a given knockout mouse strain is investigated.

Future directions

The streptomycin mouse model is ideally suited for studying innate and adaptive mucosal immune defense mechanisms and bacterial virulence factors contributing to (or limiting) disease. Variants of this model, like gnotobiotic mice and the immunization-challenge protocol, have expanded the possible applications to the analysis of microbiota function and the study of adaptive sIgA responses. The following topics might be of particular importance for future work.

Salmonella infection biology

1. Deciphering the timing of the multilayered network of cytokines triggering, sustaining and controlling the cellular

responses in the infected mucosa. Novel imaging techniques will be of importance.

2. How do the different *Salmonella* virulence factors act in concert?

3. Comprehensive analysis of the environmental factors defining the 'inflamed gut' ecosystem. What are the effects on the host, the microbiota and the pathogen?

Mucosal immunology

1. How are pathogens and microbiota handled by the phagocytes of the mucosal immune system?

2. Mechanisms driving remission from acute mucosal inflammation.

3. Elicitation of mucosal immune responses against polymeric glyco-antigens.

4. The generation, mechanisms and longevity of adaptive mucosal immune responses raised against a defined 'neo-antigen'.

Functional analysis of the microbiota

1. What is the cellular and the molecular basis of colonization resistance?

2. How does the microbiota recover after antibiotic- or pathogen inflicted insult?

The streptomycin mouse model will provide an excellent system for addressing these questions.

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