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Lipocalin-2 resistance of *Salmonella enterica* serotype Typhimurium confers an advantage during life in the inflamed intestine

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SUMMARY

Salmonella enterica serotype Typhimurium thrives in the lumen of the acutely inflamed intestine, which suggests that this pathogen is resistant to antimicrobials encountered in this environment. However, the identity of these antimicrobials and the corresponding bacterial resistance genes remains elusive. Here we show that enteric infection with *S. Typhimurium* evoked marked interleukin (IL)-22/IL-17 mediated induction in intestinal epithelial cells of lipocalin-2, an antimicrobial protein that prevents bacterial iron acquisition. Lipocalin-2 accumulated in the intestinal lumen of rhesus macaques during *S. Typhimurium* infection. Resistance to lipocalin-2, mediated by the *iroBCDE iroN* locus, conferred a competitive advantage upon the *S. Typhimurium* wild-type in colonizing the inflamed intestine of wild-type, but not of lipocalin-2 deficient mice. These data support that resistance to lipocalin-2 defines a specific adaptation to growth in the inflamed intestine.

INTRODUCTION

Salmonella enterica serotype Typhimurium (*S. Typhimurium*) causes a localized gastroenteritis in humans, characterized by acute intestinal inflammation and diarrhea. The pathogen encodes two type III secretion systems (T3SS-1 and T3SS-2) that are important for eliciting intestinal inflammation (Hapfelmeier et al., 2005; Tsois et al., 1999). The initiation of inflammatory responses in tissue requires direct contact between bacteria and host cells,

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including epithelial cells, macrophages and dendritic cells. Macrophages and dendritic cells infected with *S. Typhimurium* are a source of cytokines, including interleukin [IL]-23 and IL-18, that help to amplify responses in tissue (Godinez et al., 2009; Srinivasan et al., 2007). For example, IL-23 stimulates T cells in the intestinal mucosa to produce IL-17 and IL-22 (Godinez et al., 2009), two cytokines whose expression is among the ones most prominently induced during *S. Typhimurium* infection (Godinez et al., 2008; Raffatellu et al., 2008). The cytokine storm ensuing from the amplification of inflammatory responses in tissue results in the activation of antimicrobial responses in the intestinal mucosa.

One important component of the antimicrobial response induced by the IL-23/IL-17 axis is the recruitment of neutrophils (Godinez et al., 2008; Godinez et al., 2009; Raffatellu et al., 2008). The extravasation of neutrophils into the mucosa provides a formidable antibacterial defense to prevent *S. Typhimurium* dissemination. Support for this notion comes from clinical data, showing that neutropenia is a risk factor for bacteremia with non-typhoidal *Salmonella* serotypes (Noriega et al., 1994). Thus *S. Typhimurium* appears to be susceptible to this arm of the host defense.

A second component of the antimicrobial response is the production of antimicrobial proteins in the intestinal mucosa (Godinez et al., 2009; Raffatellu et al., 2008; Zheng et al., 2008), whose release into the intestinal lumen may be responsible for the dramatic changes in the microbiota observed during inflammation (Lupp et al., 2007; Stecher et al., 2007). The cytokines IL-17 and IL-22 are required for the production in the inflamed intestine of antimicrobials (Godinez et al., 2009; Raffatellu et al., 2008), including lipocalin-2, a protein that inhibits bacterial growth by interfering with the acquisition of an essential nutrient, iron (Berger et al., 2006; Flo et al., 2004; Goetz et al., 2002). However, the cells targeted by IL-17 and IL-22 *in vivo* to induce production of these antimicrobials have not been identified. *S. Typhimurium* appears to be resistant against this arm of the host defense, because its numbers in the intestinal lumen increase dramatically during inflammation, resulting in increased fecal oral transmission (Barman et al., 2008; Lawley et al., 2008; Stecher et al., 2007). We thus hypothesized that, in addition to virulence mechanisms to induce intestinal inflammation, *S. Typhimurium* should possess virulence mechanisms to survive the ensuing antimicrobial responses in the intestinal lumen. While the virulence factors involved in triggering intestinal inflammation, such as T3SS-1 and T3SS-2, are well characterized, the identity of the antimicrobial responses potentially encountered in the lumen of the inflamed intestine and the identity of the corresponding bacterial resistance genes are currently unknown.

To gain further insights into this “inflammation-adapted” pathogenic lifestyle of *S. Typhimurium*, we set out to identify antimicrobials released by intestinal epithelial cells into the intestinal lumen during inflammation. After identifying one such antimicrobial we determined whether the corresponding *S. Typhimurium* resistance genes conferred an advantage during bacterial growth in the inflamed intestine.

RESULTS

Gene expression profile induced by IL-17 and IL-22 in human intestinal model epithelia

To identify antimicrobial responses elicited by IL-17 and IL-22 in the intestinal epithelium, human colonic cancer epithelial (T84) cells were polarized and basolaterally stimulated with IL-17 or IL-22. Expression of *CCL20*, a chemokine gene known to be induced by IL-17 in lung epithelial cells (Kao et al., 2005) was found to be induced optimally 4 hours after stimulation with IL-17 or IL-22 (data not shown). To determine the complete molecular profile of epithelial responses, RNA from four replicates was isolated 4 hours after stimulation of T84 cells with IL-22 or IL-17 and global gene expression profiles were elucidated by microarray analysis.

In general, IL-22 produced more robust responses in T84 cells than IL-17 (Supplementary Figure 1). For example, the number of genes up regulated 2-fold or more was 849 for IL-22 treatment compared to only 62 for IL-17 treatment. A table containing a complete listing of genes with significantly ($P < 0.05$) altered expression in T84 epithelial cells after stimulation with IL-17 or IL-22 can be accessed at the Gene Expression Omnibus database [GSE11345].

Meta analysis of *in vitro* and *in vivo* gene expression profiles suggests a contribution of epithelial cells to host defense responses during inflammation

To identify those antimicrobial responses induced *in vitro* in T84 cells that are also observed in the inflamed intestine *in vivo*, we performed meta analysis of the overlap in up regulated gene transcription (>2 fold) between T84 cells stimulated with IL-22 and the ileal mucosa of rhesus macaques infected with *S. Typhimurium* (Figure 1). The *in vivo* gene expression profile analyzed in this study had previously been generated using an adult healthy rhesus macaque that had undergone ligated ileal loop surgery (Raffatellu et al., 2008). Two ligated ileal loops had been inoculated with either sterile culture medium (mock infection) or with *S. Typhimurium* and 5 hours later both loops had been surgically removed to isolate RNA for gene expression profiling (Raffatellu et al., 2008). Meta analysis revealed a substantial overlap between transcripts whose levels were increased in the ileal mucosa of a rhesus macaque during *S. Typhimurium* infection with those whose expression was induced in human T84 epithelial cells upon stimulation with IL-22 (302 transcripts) (Figure 1A). A functional category that was statistically over-represented included genes involved in defense responses, encoding antimicrobials (*LCN2*, *NOS2* and *MUC4*) and cytokines (*CCL20*) whose expression was increased most dramatically both *in vitro* in IL-22 treated T84 cells and during *S. Typhimurium* infection *in vivo* (Figure 1A). These data supported the hypothesis that IL-22 induced expression of antimicrobials in epithelial cells during *S. Typhimurium* infection in the intestinal mucosa.

IL-17 and IL-22 synergize to induce lipocalin-2 production in intestinal epithelial cells

Transcripts encoding the antimicrobial lipocalin-2 were among the ones most prominently increased both *in vitro* after IL-22 stimulation and *in vivo* after *S. Typhimurium* infection (Figure 1 A). Thus, after validating results from gene expression profiling for selected genes by real-time PCR (Supplementary Figure 2), we sought to further investigate whether intestinal epithelial cells may be a source of lipocalin-2 during *S. Typhimurium* infection. First, we verified that lipocalin-2 is an antimicrobial produced by intestinal epithelial cells. Quantification of changes in gene expression by real-time PCR confirmed that stimulation of T84 cells with IL-22 resulted in an induction of *LCN2* expression (7.9 fold) (Figure 1B). Stimulation with IL-17 alone did not alter *LCN2* transcription (1.3 fold change) but further increased *LCN2* transcription when added in combination with IL-22 (17 fold). We next determined protein secretion by ELISA. Non-stimulated T84 cells secreted a small amount of lipocalin-2 apically (approximately 2ng/ml), while the amount detected in the basolateral compartment was negligible. Stimulation of T84 cells with IL-22, but not with IL-17, resulted in apical and basolateral secretion of lipocalin-2 at levels above those detected with non-stimulated controls. Stimulation with both IL-17 and IL-22 resulted in a significant ($P < 0.05$) further increase of lipocalin-2 secretion both apically and basolaterally, reaching substantial concentrations (approximately 20ng/ml) in both compartments (Figure 1C). In contrast, *CCL20* was secreted exclusively into the basolateral compartment after cytokine stimulation of T84 cells (Supplementary figure 2). Although IL-17 by itself did not markedly alter *LCN2* transcription, our data suggested that this cytokine synergized with IL-22 in controlling production of lipocalin-2 in the intestinal epithelium.

Because lipocalin-2 was highly induced during *S. Typhimurium* infection *in vivo*, we next investigated whether expression of *LCN2* was induced either by cytokines (i.e. IL-17 and

IL-22) present in the inflamed intestine, by direct contact of bacteria with epithelia cells or by a combination of both mechanisms. Basolateral stimulation of T84 cells with purified flagellin or infection of T84 cells with the *S. Typhimurium* wild-type strain, an invasion deficient strain (*invA* mutant) or a non-flagellated strain (*fliC fljB* mutant) did not result in marked induction in *LCN2* expression (Figure 1D). Treatment of T84 cells with IFN- γ did not increase *LCN2* transcription. The marked increase in *LCN2* transcription observed after basolateral stimulation of T84 cells with IL-17 and IL-22 was modestly increased by basolateral stimulation with purified flagellin ($P = 0.03$) or by *S. Typhimurium* infection ($P = 0.004$). These data suggested that induction of *LCN2* transcription was mediated largely through stimulation with IL-17 and IL-22. Although direct interaction between bacteria and host cells was not sufficient to induce *LCN2* transcription, these interactions could further increase *LCN2* mRNA levels induced by treatment with IL-17 and IL-22.

Lipocalin-2 produced by epithelial cells after stimulation with IL-17 and IL-22 inhibits bacterial growth

To investigate the biological significance of the IL-17/IL-22-mediated lipocalin-2 production by intestinal epithelial cells, we determined whether the protein was produced at levels that exhibited an antimicrobial activity. Lipocalin-2 specifically binds enterochelin, a small molecular weight iron chelator (siderophore) produced by many members of the *Enterobacteriaceae*. As a result, lipocalin-2 exhibits a bacteriostatic effect on bacteria that depend exclusively on enterochelin to acquire iron, an essential trace element, during growth in the host (Berger et al., 2006; Flo et al., 2004; Goetz et al., 2002). The *iroBCDE iroN* gene cluster of *S. Typhimurium* encodes proteins involved in the biosynthesis and uptake of salmochelin, a glycosylated derivative of enterochelin (Baumler et al., 1998; Baumler et al., 1996; Bister et al., 2004; Hantke et al., 2003; Rabsch et al., 1999; Zhu et al., 2005). Salmochelin is not bound by lipocalin-2 and its production therefore renders *S. Typhimurium* lipocalin-2 resistant. However, in the absence of a functional *iroBCDE iroN* gene cluster, *S. Typhimurium* produces enterochelin as its sole siderophore and is lipocalin-2 sensitive (Crouch et al., 2008; Fischbach et al., 2006).

Growth of the *S. Typhimurium* wild-type (lipocalin-2 resistant) and a *S. Typhimurium iroBC* mutant (lipocalin-2 sensitive) were compared in medium collected either from non-stimulated T84 cells (control) or from T84 cells after stimulation with IL-17 and IL-22 (Figure 2). No differences between the *S. Typhimurium* wild-type and the *iroBC* mutant were observed in control media, however growth of the *iroBC* mutant was significantly ($P < 0.05$) reduced in media in which lipocalin-2 production had been elicited by stimulation with IL-17 and IL-22 (Figure 2A). These data suggested that the amount of lipocalin-2 produced by intestinal epithelial cells upon IL-17/IL-22 stimulation was sufficient to reduce growth of the *iroBC* mutant *in vitro*.

A mutation in *iroBC* did not impair bacterial growth in rich media (Luria-Bertani broth) (Figure 2B). Tissue culture medium was spiked with lipocalin-2 to confirm that the presence of this antimicrobial was responsible for the growth defect observed with the *iroBC* mutant. The presence of lipocalin-2 significantly ($P < 0.05$) reduced growth of the *iroBC* mutant, while growth of the *S. Typhimurium* wild-type was not affected (Figure 2C). Complementation of the *iroBC* mutant with the cloned *iroBCDE iroN* gene cluster restored resistance to lipocalin-2, as indicated by equal growth in tissue culture medium in the presence or absence of lipocalin-2 (Figure 2C). The lipocalin-2 mediated growth inhibition of the *iroBC* mutant could be prevented when iron was supplied in form of ferrioxamine B (iron-desferal), a siderophore that can be internalized by the FoxA outer membrane receptor of *S. Typhimurium* (Figure 2C). Collectively, these data suggested lipocalin-2 inhibited growth of the *iroBC* mutant through iron sequestration.

S. Typhimurium infection induces *LCN2* expression in intestinal epithelial cells of rhesus macaques and secretion of lipocalin-2 into the intestinal lumen

To investigate the *in vivo* relevance of lipocalin-2 production by T84 cells, we examined the contribution of epithelial cells expressing this antimicrobial protein during *S. Typhimurium* infection in a relevant host. To this end, we first localized the *LCN2* transcripts in the ileal mucosa of rhesus macaques using *in situ* hybridization (Figure 3). *LCN2* transcripts localized to epithelial cells lining the villi and crypts, with intense staining in tissue from *S. Typhimurium*-infected ileal loops with a *LCN2*-specific cDNA probe (Figure 3A). These data suggested that *LCN2* was mainly expressed by intestinal epithelial cells during *S. Typhimurium* infection.

Next, we determined the absolute number of *LCN2* transcripts using quantitative real-time PCR. For each rhesus macaque (n= 4), a tissue sample from a mock-infected loop and from a *S. Typhimurium* infected loop were processed for mRNA isolation. Transcripts encoding β -actin (*ACT1*) were detected at similar levels in both treatment groups, averaging approximately 20,000 copies/ng RNA in samples from mock-infected and *S. Typhimurium*-infected loops (Figure 3B). Based on theoretical assumptions about the RNA content of 10^6 cells (5 – 8 μ g) (Ausubel et al., 1994), these data would predict that *ACT1* was present on average in 100 – 160copies/cell. Our data were thus within the range of previous measurements by real-time PCR indicating that human leukocytes contain between 84 and 491copies of *ACT1* per cell (Lupberger et al., 2002). *LCN2* transcripts averaged 1,400 copies/ng RNA in samples from mock-infected loops, but the copy number was significantly ($P = 0.03$) increased after *S. Typhimurium* infection, averaging 48,000 copies/ng RNA. Collectively, these data suggested that during *S. Typhimurium* infection, the *LCN2* gene was expressed at a very high level in the epithelium of the ileal mucosa.

We next studied the production of lipocalin-2 *in situ* using immunohistochemistry (Figure 3C–E). Rabbit polyclonal antibody derived from recombinant rhesus lipocalin-2 was generated. Immuno staining of formalin fixed tissue revealed that after *S. Typhimurium* infection, lipocalin-2 was abundant in intestinal epithelial cells and in the underlying lamina propria. These data suggested that lipocalin-2 protein is produced and secreted in tissue during the course of an infection with *S. Typhimurium*. Next, we wanted to detect the amount of lipocalin-2 secreted into the intestinal lumen *in vivo*. An ELISA was developed to detect the amount of lipocalin-2 secreted into the luminal fluid previously collected from ligated ileal loops of rhesus macaques 8 hours after inoculation with *S. Typhimurium* (n=4) or sterile culture medium (n=4). Luminal contents of *S. Typhimurium* infected loops contained on average 419ng of lipocalin-2, which was significantly higher ($P = 0.007$) than the 64ng lipocalin-2 detected in luminal contents of mock-infected loops (Figure 3F). Collectively, these data identified lipocalin-2 production as an antimicrobial response encountered in the lumen of the inflamed intestine.

Lipocalin-2 resistance confers an advantage during growth of *S. Typhimurium* in the inflamed intestine

After identifying lipocalin-2 as an antimicrobial response encountered in the intestinal lumen, we wanted to determine whether the corresponding *S. Typhimurium* resistance genes would confer an advantage during bacterial growth in the inflamed intestine. To establish cause and effect using lipocalin-2 deficient animals, we used the streptomycin pretreated mouse model for these studies. *S. Typhimurium* infection of streptomycin pretreated mice results in acute inflammation of the cecal mucosa (Barthel et al., 2003), which is accompanied by overgrowth of *S. Typhimurium* in the lumen of the large intestine (Que et al., 1986; Stecher et al., 2007).

We first investigated whether inactivation of *iroN*, encoding the IroN outer membrane salmonchelin receptor protein, would reduce the ability of *S. Typhimurium* to grow in the intestinal lumen of streptomycin pretreated wild-type mice (C57BL/6) (Figure 4). The *iroN* mutant was sensitive to lipocalin-2 mediated iron deprivation *in vitro* (Figure 2C). At 48 hours after infection of streptomycin pretreated mice, the *iroN* mutant was recovered in reduced numbers from colon contents than the *S. Typhimurium* wild-type strain (Figure 4A). However, there was a large animal-to-animal variation in bacterial numbers recovered from colon contents. We next investigated whether a lipocalin-2 sensitive *S. Typhimurium* mutant (*iroN* mutant) was able to compete with the *S. Typhimurium* wild type *in vivo*. Using this experimental design, the ability of the *iroN* mutant and the wild type to colonize the intestine is compared in each individual animal, thereby reducing the effect of animal-to-animal variation. Streptomycin pretreated wild-type mice were inoculated intragastrically with sterile LB broth (mock infection) or with a 1:1 mixture of the *S. Typhimurium* wild type and the *iroN* mutant. Compared to mock-infected mice, mice inoculated with the *S. Typhimurium* strain mixture exhibited markedly increased transcript levels of cytokine genes, including *Il17*, *Il22*, keratinocyte-derived cytokine (*Kc*, *Cxcl1*) and tumor necrosis factor α (*Tnfa*) at 48 hours after infection (Figure 5A–D). Furthermore, *S. Typhimurium* infection caused marked inflammatory changes in the submucosa and mucosa (Figure 6). These data confirmed that *S. Typhimurium* infection of streptomycin-pretreated mice was associated with acute cecal inflammation. Importantly, transcript levels of *Lcn2* were markedly increased (53-fold) in *S. Typhimurium*-infected mice compared to mock-infected mice (Figure 7A). Expression of lipocalin-2 in the cecal mucosa was assessed at the protein levels using Western blot. While no lipocalin-2 was detectable in the cecal mucosa of mock-infected mice, the protein was highly abundant 48 hours after *S. Typhimurium* infection (Figure 7B). The *S. Typhimurium* wild type was recovered in 7-fold higher numbers from intestinal contents 48 hours after infection than the *iroN* mutant (Figure 4B), suggesting that IroN-mediated salmonchelin uptake constituted an advantage during growth in the inflamed cecum.

To investigate whether the presence of the *iroN* gene confers an advantage only in the presence of intestinal inflammation, we repeated the above experiments with *S. Typhimurium* strains that were unable to elicit this host response. The induction of cecal inflammation in streptomycin-pretreated mice requires the presence of functional T3SS-1 and T3SS-2 secretion systems (Hapfelmeier et al., 2005). We constructed a *S. Typhimurium* strain carrying mutations that prevent functionality of T3SS-1 (*invA*) and T3SS-2 (*spiB*). Streptomycin pretreated wild-type mice (C57BL/6) were inoculated intragastrically with sterile LB broth (mock infection) or with a 1:1 mixture of the *S. Typhimurium invA spiB* mutant and a *S. Typhimurium invA spiB iroN* mutant. Compared to mock infected mice, no marked increases in cytokine transcription (*Il17*, *Il22*, *Kc*, and *Tnfa*) were detected in the cecal mucosa of mice infected with a mixture of the *S. Typhimurium invA spiB* mutant and the *invA spiB iroN* mutant (Figure 5) and no marked inflammatory changes were detected microscopically (Figure 6). Increased expression of *Lcn2* transcription in the cecal mucosa was not detected by real-time PCR and little lipocalin-2 expression was detected by Western blot (Figure 7). These data confirmed that infection with T3SS-1/T3SS-2 deficient *S. Typhimurium* strains does not trigger marked cecal inflammation in streptomycin-pretreated mice. Importantly, the *S. Typhimurium invA spiB* mutant and the *invA spiB iroN* mutant were recovered in equal numbers from intestinal contents 48 hours after infection (Figure 4B). These data suggested that in the absence of intestinal inflammation, the IroN-mediated salmonchelin uptake did not provide a significant selective advantage during growth in the intestine.

To investigate whether intestinal inflammation provides a selective advantage for *S. Typhimurium* strains carrying the IroN outer membrane receptor because expression of lipocalin-2 is highly induced in the inflamed intestine, above experiments were repeated using lipocalin-2-deficient mice. Streptomycin pretreated lipocalin-2-deficient mice were inoculated

intra-gastrically with sterile LB broth (mock infection) or with a 1:1 mixture of the *S. Typhimurium* wild type and the *S. Typhimurium iroN* mutant. Inoculation with a mixture of the *S. Typhimurium* wild type and the *S. Typhimurium iroN* mutant resulted in acute cecal inflammation, as indicated by markedly increased transcript levels of *Il17*, *Il22*, *Kc*, and *Tnfa* at 48 hours after infection and inflammatory changes in tissue (Figures 5 and 6). As expected, lipocalin-2 expression was detected neither by real-time PCR nor by Western blot in the ceca of any of the lipocalin-2-deficient mice (Figure 7). Importantly, the *S. Typhimurium* wild type and the *iroN* mutant were recovered in similar numbers from intestinal contents 48 hours after infection (Figure 4B). Furthermore, the ratio of wild type to *iroN* mutant (competitive index) recovered from lipocalin-2-deficient mice was significantly lower than that determined for wild type mice ($P < 0.05$). These data suggested that *iroN* confers a selective advantage during growth in the inflamed intestine because the encoded salmochelin receptor IroN mediates resistance to lipocalin-2.

DISCUSSION

One host strategy for controlling colonization of unwanted microbes is to limit the availability of iron, an essential nutrient, by sequestering this trace element both systemically by hepcidin-mediated acute phase responses (Ganz, 2006; Nemeth and Ganz, 2006), and locally by high affinity iron binding proteins, including lactoferrin secreted by neutrophils at sites of inflammation (Ong et al., 2006). To overcome this iron-withholding defense, bacteria release high-affinity iron chelators, termed siderophores, that can remove iron from host proteins and are subsequently taken up by specialized bacterial transport systems (Braun et al., 1998). The siderophore produced by most members of the *Enterobacteriaceae* is enterochelin, a cyclic trimer of dihydroxybenzoylserine (O'Brien and Gibson, 1970). The host can limit the ability of bacteria to use enterochelin for iron acquisition by secreting lipocalin-2, a protein whose production in tissue is induced by IL-22 (Aujla et al., 2008) and IL-17 (Conti et al., 2009; Raffatellu et al., 2008) during inflammation. Expression of *LCN2* mRNA by the inflamed intestinal epithelium occurs in the human colon during inflammatory bowel disease (Nielsen et al., 1996), while murine *Lcn2* mRNA is present in mouse colonic epithelium during dextran sodium sulfate-induced colitis (Playford et al., 2006). Here we show that IL-17 and IL-22 act in concert to induce *LCN2* transcription in T84 colonic epithelial cells. Furthermore, *LCN2* transcription was induced by *S. Typhimurium* infection in the intestinal epithelium of rhesus macaques, resulting in secretion of lipocalin-2 into the intestinal lumen. These data suggested that lipocalin-2 is an antimicrobial encountered in the lumen of the inflamed intestine.

Lipocalin-2 specifically binds and inactivates enterochelin, resulting in a bacteriostatic activity for some commensal bacteria, such as *Escherichia coli* (Berger et al., 2006; Flo et al., 2004; Goetz et al., 2002). The *iroBCDE iroN* gene cluster of *S. Typhimurium* encodes proteins involved in the biosynthesis and uptake of salmochelin, a glycosylated derivative of enterochelin (Bister et al., 2004; Hantke et al., 2003; Zhu et al., 2005). Salmochelin is not bound by lipocalin-2 and its production therefore confers lipocalin-2 resistance (Crouch et al., 2008; Fischbach et al., 2006). Here we show that lipocalin-2 resistance conferred a specific benefit during growth of *S. Typhimurium* in the inflamed cecum of mice. The streptomycin pretreated mouse model has limitations, because exudative intestinal inflammation is induced artificially by antibiotic treatment. However, our results show that lipocalin-2 production is also a prominent host response to *S. Typhimurium* infection in the intestinal mucosa of rhesus macaques, a highly relevant model for human gastroenteritis. Our results suggest that acquisition of the *iroBCDE iroN* gene cluster by *S. Typhimurium* may represent a specific adaptation to life in the inflamed intestine. A potential role of lipocalin-2 resistance in promoting colonization of the inflamed nasal mucosa has recently been proposed for *Streptococcus pneumoniae* and *Haemophilus influenzae* (Nelson et al., 2005).

The main virulence factors involved in inducing intestinal inflammation, T3SS-1 and T3SS-2 (Hapfelmeier et al., 2005; Tsolis et al., 1999), were acquired by horizontal gene transfer early after divergence of the genus *Salmonella* from the *E. coli* lineage and are present in all members of the species *S. enterica* (Hensel et al., 1997; Li et al., 1995; Ochman and Groisman, 1996). It seems reasonable to assume that to ensure survival, acquisition of T3SS-1 and T3SS-2 had to be accompanied by acquisition of genes that confer resistance to antimicrobials encountered in the inflamed intestine. Consistent with this idea, the *iroBCDE iroN* gene cluster is present in all members of the species *S. enterica* but is absent from the genomes of commensal *E. coli* strains (Baumler et al., 1997; Baumler et al., 1998). The only *E. coli* isolates in which the *iroBCDE iroN* gene cluster is present are uropathogenic *E. coli* (UPEC) (Bauer et al., 2002; Sorsa et al., 2003). This distribution is consistent with the concept that the *iroBCDE iroN* gene cluster represents an adaptation to life at inflamed mucosal surfaces, which is an environment encountered by both UPEC (cystitis) and *S. Typhimurium* (gastroenteritis).

In addition to overcoming lipocalin-2 mediated iron withholding, *S. Typhimurium* likely needs to resist many other antimicrobial defense mechanisms encountered in the inflamed intestine. Candidate antimicrobials whose transcripts are prominently induced in the intestinal mucosa during *S. Typhimurium* infection include inducible nitric oxide synthase (iNOS, encoded by *NOS2*), regenerating islet-derived 3 gamma (RegIII γ), calprotectin A, mucin (*MUC4*), indoleamine 2,3-dioxygenase (IDO), dual oxidase (Duox) and defensins (Godinez et al., 2009; Raffatellu et al., 2007; Raffatellu et al., 2008; Zheng et al., 2008). Here we show that IL-22 induced expression of *NOS2* and *MUC4* in human intestinal epithelial cells, suggesting a possible cellular source for the high mRNA levels of these genes observed in the ileal mucosa of rhesus macaques during *S. Typhimurium* infection (Raffatellu et al., 2008). In mice lacking iNOS and NADPH oxidase, *S. Typhimurium* is recovered in reduced numbers from intestinal contents at 48 hours after infection, although this difference is not statistically significant (Ackermann et al., 2008). Research on the potential role of *S. Typhimurium* resistance mechanisms against the various antimicrobials encountered in the inflamed intestine represents an exciting area for future study.

MATERIALS AND METHODS

Tissue culture assays

T84 cells were grown in a 1:1 mixture of DMEM/F12 and 10% Fetal calf serum (Gibco). To achieve polarization, cells were seeded on the apical compartment in 12-mm-diameter Transwell plate (Corning Costar) and utilized when a TER of at least 1,500 Ω .cm² was reached.

Stimulation with cytokines

Recombinant human IL-17, IL-22 and IFN- γ were obtained from R&D systems and utilized at the following final concentrations: IL-17, 100 ng/ml; IL-22, 100 ng/ml; IFN- γ , 40 ng/ml. Each stimulation experiment was performed a minimum of four times.

Gene expression profiling

Gene expression profiles (GEO accession number GSE11345) in polarized T84 cells were monitored utilizing the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). For each treatment group, total RNA from 4 tissue culture wells was analyzed. A minimum 2-fold difference in transcription between control and experimental samples was used as criteria for identifying a “change” in gene expression (P value < 0.05; 95% confidence).

Real-time PCR

For analysis of gene expression by real-time PCR, total RNA was extracted with Tri-reagent (Molecular Research Center) and processed as described earlier (Raffatellu et al., 2007). Real-time PCR was performed using SYBR Green (Applied Biosystems) and the 7900HT Fast Real-Time PCR System. The data were analyzed using the comparative delta-Ct method (Applied Biosystems). Analysis of the copy number of rhesus *LCN2* was performed by comparing crossing points of experimental cDNA to standard curves generated from a plasmid that contained a fragment of rhesus *LCN2* cDNA (Hornsby et al., 2008). A list of the real-time primers used in this study is provided in Supplementary Table 1.

Bacterial strains and culture conditions

IR715 is a fully virulent, nalidixic acid resistant derivative of *S. Typhimurium* wild-type isolate ATCC 14028. Construction of IR715 derivatives carrying mutations in *iroBC*, *iroN*, *invA*, or *spiB* is described in the supplementary Material and Methods. A complete list of strains, plasmids and primers used for cloning and strain construction is provided in Supplementary Tables 2 and 3. All strains were grown aerobically at 37°C in Luria-Bertani (LB) broth unless otherwise noticed.

Bacterial growth in cell culture supernatant

S. Typhimurium wild-type and *iroBC* mutant were grown overnight in iron-limiting conditions (Nutrient Broth supplemented with 0.2 mM 2,2'-dipyridyl) at 37°C with aeration over night. Approximately 1000 CFU were inoculated into tissue culture medium (DMEM/F12), DMEM/F12 containing human lipocalin-2 (100ng/ml, R&D Systems), DMEM/F12 containing human lipocalin-2 (100ng/ml) and ferrioxamine B (1µg/ml), or the media collected from the basolateral side of transwell plate 24 hours after stimulation with IL-17 and IL-22 or mock control. CFU were enumerated by plating serial dilution 5 hours after inoculation. The experiment was performed three times.

Immunoblot

Total protein was extracted from mouse cecum using Tri-Reagent (Molecular Research Center). Total protein (15 µg) was resolved by SDS-PAGE and transferred to a PVDF membrane. Detection of mouse tubulin was performed with a primary rabbit polyclonal antibody (Cell Signaling Technology) and detection of mouse lipocalin-2 was performed with a primary rabbit polyclonal antibody (R&D systems). As secondary antibody, a secondary goat-anti-rabbit conjugate to horseradish peroxidase (HRP) (Jackson) was used.

Enzyme-linked ImmunoSorbent Assay

Supernatant from both the apical and the basolateral compartments of polarized T84 cells was collected 24 hours post-stimulation. Secretion of CCL20 and lipocalin-2 was detected by using commercially available kits (R&D systems).

In situ hybridization

Localization of rhesus mRNA transcripts in formalin fixed, paraffin embedded ileal sections was performed as described earlier (Santos et al., 2002). Probes for target genes were amplified from rhesus cDNA by using the primers reported in Supplementary table 1 and cloned into PCR2.1 (Invitrogen). Each probe (*LCN2* and plasmid control) was biotin-labeled by using the NEBlot Phototype Kit according to instructions from the manufacturer (New England Biolabs).

Detection of lipocalin-2 in tissue of rhesus macaques

Generation of an anti-rhesus lipocalin-2 rabbit polyclonal antibody is described under Supplementary Material and Methods. Immunohistochemical staining of lipocalin-2 was performed on formalin fixed, paraffin embedded ileal sections by using a rabbit polyclonal antibody derived from recombinant rhesus lipocalin-2, followed by detection with a secondary anti-rabbit IgG antibody (Vector Laboratories). Staining of an adjacent section with pre-immune rabbit serum was used as a control. Detection of lipocalin-2 in the fluid collected from ligated ileal loops of rhesus macaques is described under Supplementary Material and Methods.

Mouse experiments

We used both C57BL/6 wild-type and lipocalin-2-deficient mice (*Lcn2*^{-/-}). Generation of *Lcn2*^{-/-} mice (Berger et al., 2006) is described in the supplementary materials and methods. Groups of 6 mice were pre-treated with streptomycin (0.1 ml of a 200 mg/ml solution in sterile water) intragastrically prior to mock-infection or inoculation with a mixture of *S. Typhimurium* strains (5×10^8 CFU/animal). At 48 hours after infection, mice were euthanized and cecum was collected for isolation of mRNA and protein and for histopathological analysis. Colon contents were collected and serial 10-fold dilutions were plated for enumerating bacterial CFUs on agar plates containing the appropriate antibiotics. Data were normalized by dividing the output ratio (CFU of the wild-type/CFU of the mutant) by the input ratio (CFU of the wild-type/CFU of the mutant).

Statistical analysis

Microarray data were analyzed using model-based algorithms (Affymetrix Gene Chip Operating System software, dChip, <http://biosun1.harvard.edu/complab/dchip>) and t-tests. Differences between treatment groups in fold changes in mRNA levels measured by Real-time PCR, protein levels measured by ELISA, NO production measured by Griess assays, and bacterial numbers were analyzed by ANOVA followed by Student's t test. A *P* value equal or below 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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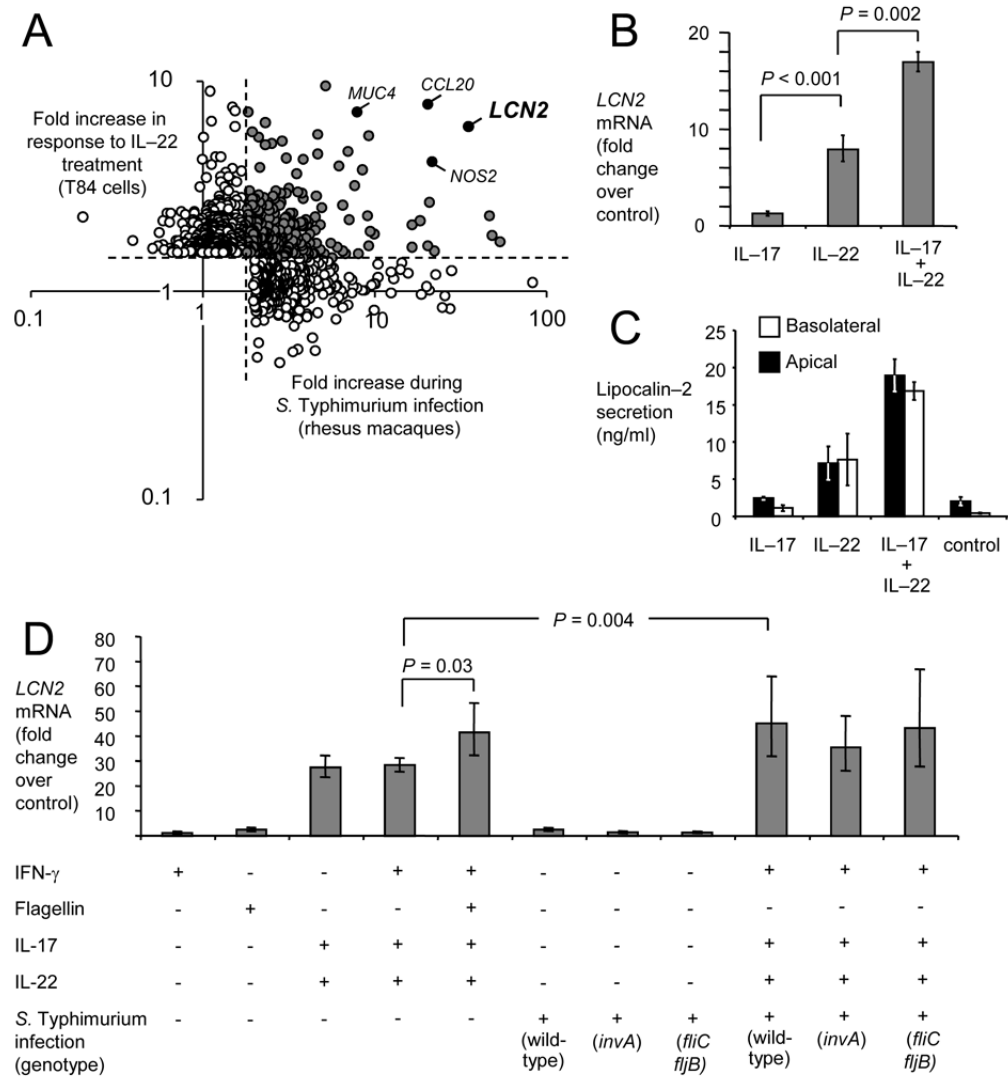


Figure 1. Expression of lipocalin-2 by polarized intestinal model epithelia upon stimulation with IL-17 and/or IL-22. (A) Meta analysis of the overlap (gray circles) between increases (>2 fold) in transcripts in the ileal mucosa of rhesus macaques during *S. Typhimurium* infection (x axis) and induction of gene expression observed in human T84 epithelial cells after treatment with IL-22 (y axis). The identity of selected genes (closed circles) is indicated. (B and D) Detection of *LCN2* expression in polarized T84 cells stimulated for 4 hours with reagents listed below each graph using quantitative real-time PCR. (C) Secretion of lipocalin-2 by T84 cells into the apical (open bars) or basolateral (closed bars) compartment detected by ELISA. (B-D) Data represent means \pm standard deviation from at least three different experiments.

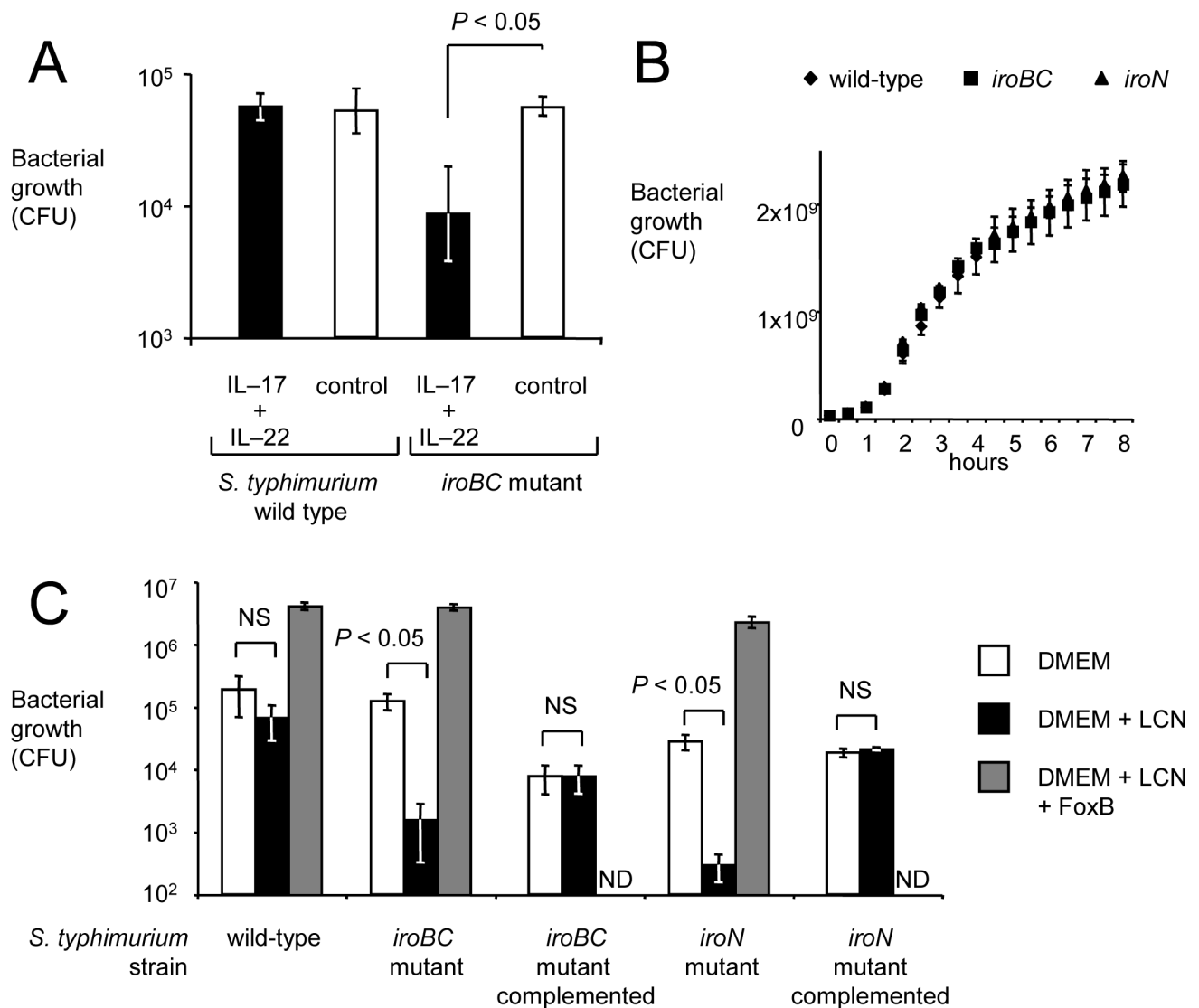


Figure 2.

The *iroBC* locus confers lipocalin-2 resistance by overcoming iron limitation. (A) Growth of the *S. Typhimurium* wild type or an isogenic *iroBC* mutant in medium from collected from polarized T84 cells that had either not been stimulated with cytokines (control, open bars) or had been stimulated with IL-17 and IL-22 for 24 hours (IL-17 + IL-22, closed bars). Bacterial numbers were determined 5 hours after inoculation with 10^3 bacteria. (B) Bacterial growth in rich medium (LB broth). (C) Bacterial growth in tissue culture medium (DMEM) supplemented with lipocalin-2 (LCN), ferrioxamine B (FoxB) or containing no supplements. ND, not determined. All data represent means \pm standard deviation from at least three different experiments.

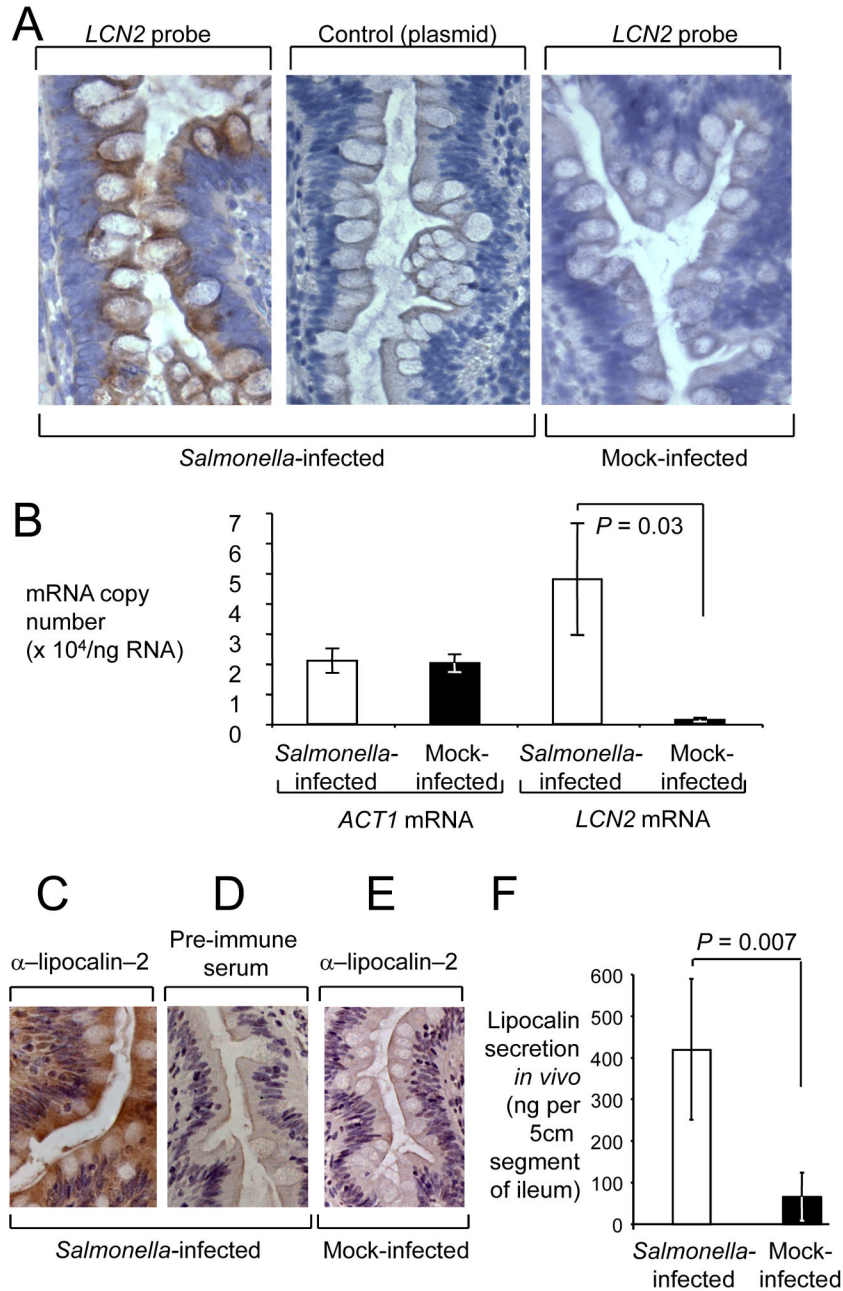


Figure 3. Spatial and quantitative analysis of lipocalin-2 expression in the ileal mucosa of rhesus macaques during *S. Typhimurium* infection. **(A)** Detection of *LCN2* transcripts (brown precipitate) in representative sections from a *S. Typhimurium*-infected loop (left panel) and a mock-infected loop (right panel) from the same animal by *in situ* hybridization. Hybridization with the empty plasmid vector used for cloning the *LCN2* probe was performed as a negative control (middle panel). All sections were counterstained with hematoxylin. **(B)** Absolute transcript levels of *LCN2* and *ACT1* in mock-infected (closed bars) and *S. Typhimurium*-infected loops (open bars) from 4 rhesus macaques. Data represent mean mRNA copy numbers per ng of RNA \pm standard error. Statistically significant ($P < 0.05$) differences are indicated

by *P* values. **(C–E)** Detection of lipocalin-2 (brown precipitate) in representative sections from a *S. Typhimurium*-infected loop (**C** and **D**) and a mock-infected loop (**E**) from the same animal using immunohistochemistry with rabbit anti-rhesus lipocalin-2 antiserum (α -lipocalin-2). Immunohistochemistry with pre-immune serum was performed as a negative control (**D**). **(F)** Lipocalin-2 secretion in mock-infected (closed bars) and *S. Typhimurium*-infected loops (open bars) from 4 rhesus macaques. Data represents the total amount of lipocalin-2 (ng) secreted into an approximately 5 cm long segment of the ileum. Statistically significant ($P < 0.05$) differences are indicated by *P* values.

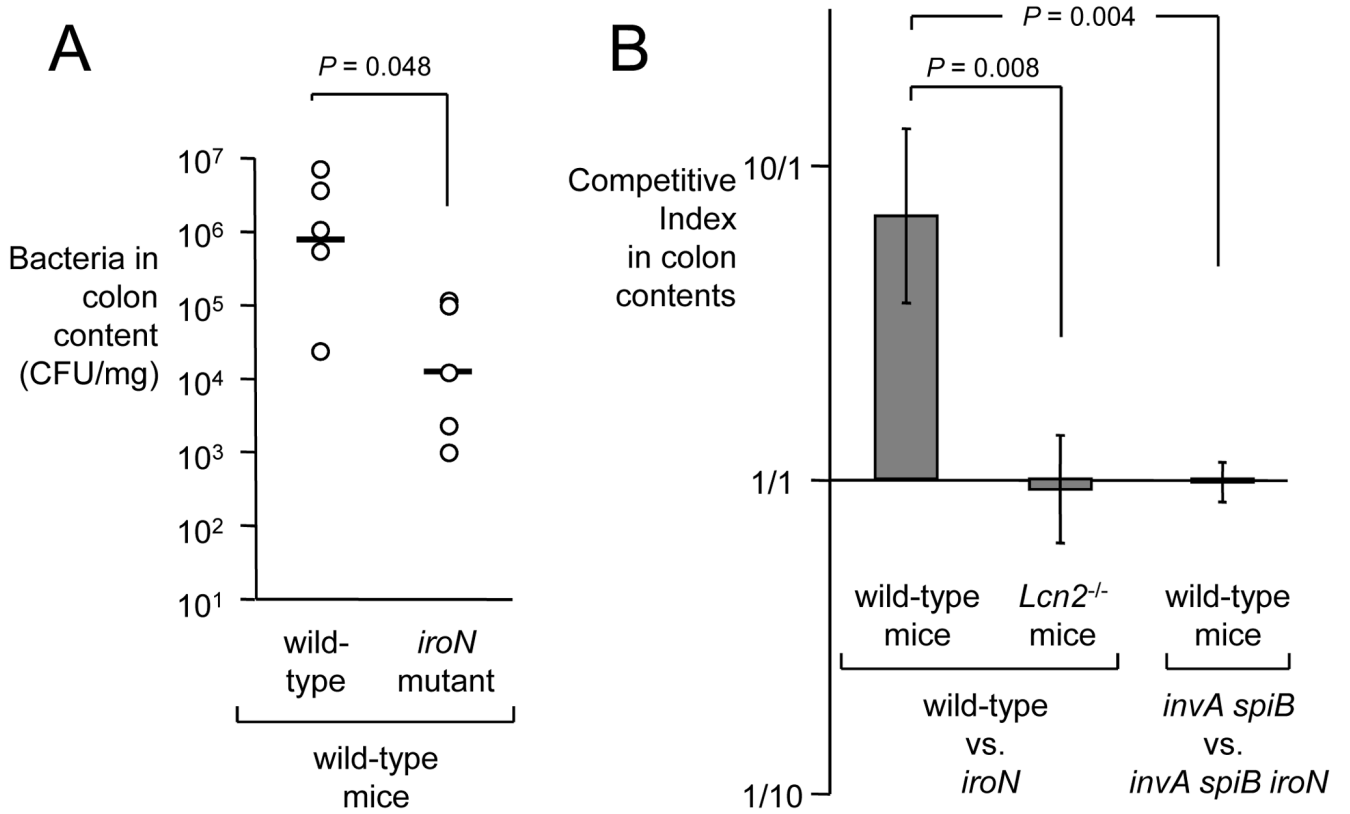


Figure 4. Growth of *iroN* deficient and *iroN* proficient *S. Typhimurium* strains in the murine intestine *in vivo*. **(A)** Recovery of *S. Typhimurium* from colon contents 48 hours after infection of mice with the *S. Typhimurium* wild-type or an *iroN* mutant. Each circle represent bacterial numbers recovered from an individual animal. Bars indicate the geometric mean. **(B)** Recovery of *S. Typhimurium* 48 hours after competitive infection with the indicated bacterial strains. Bars indicate the average competitive index (i.e. ratio of wild-type/*iroN* or ratio of *invA spiB/invA spiB iroN*) of bacteria recovered from colon contents of wild-type mice or *Lcn2*^{-/-} mice 48 hours after infection with a 1:1 mixture the respective *S. Typhimurium* strains. Data represent geometric means \pm standard error.

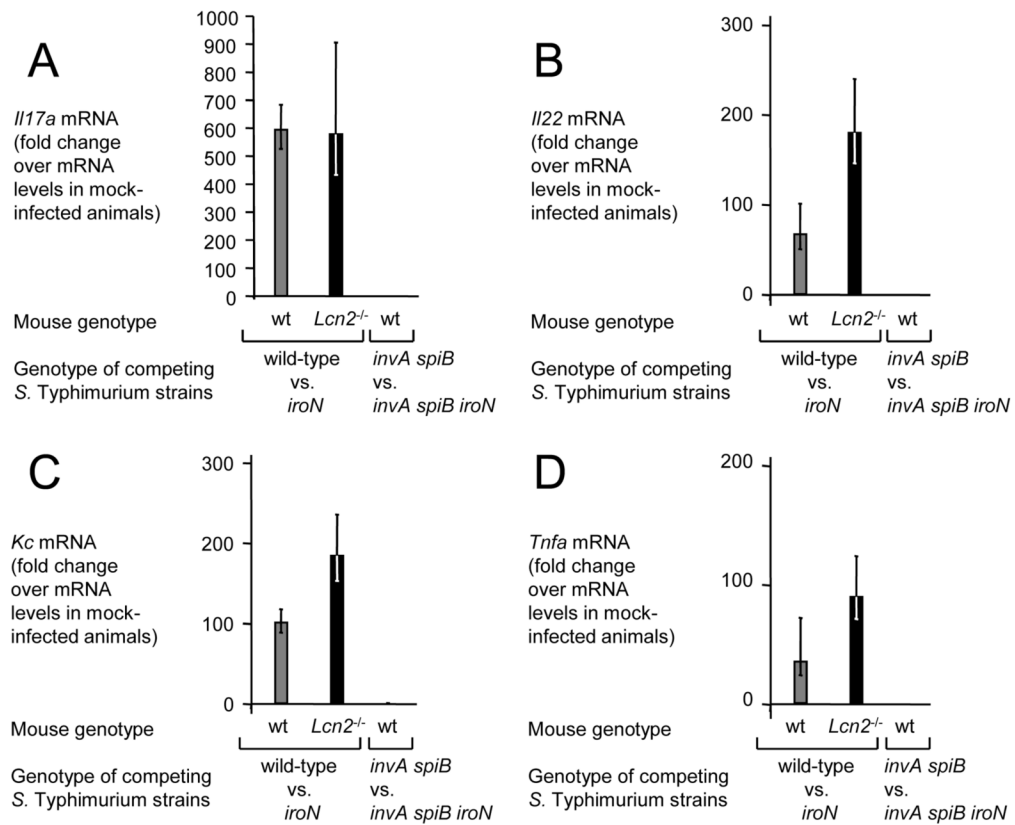
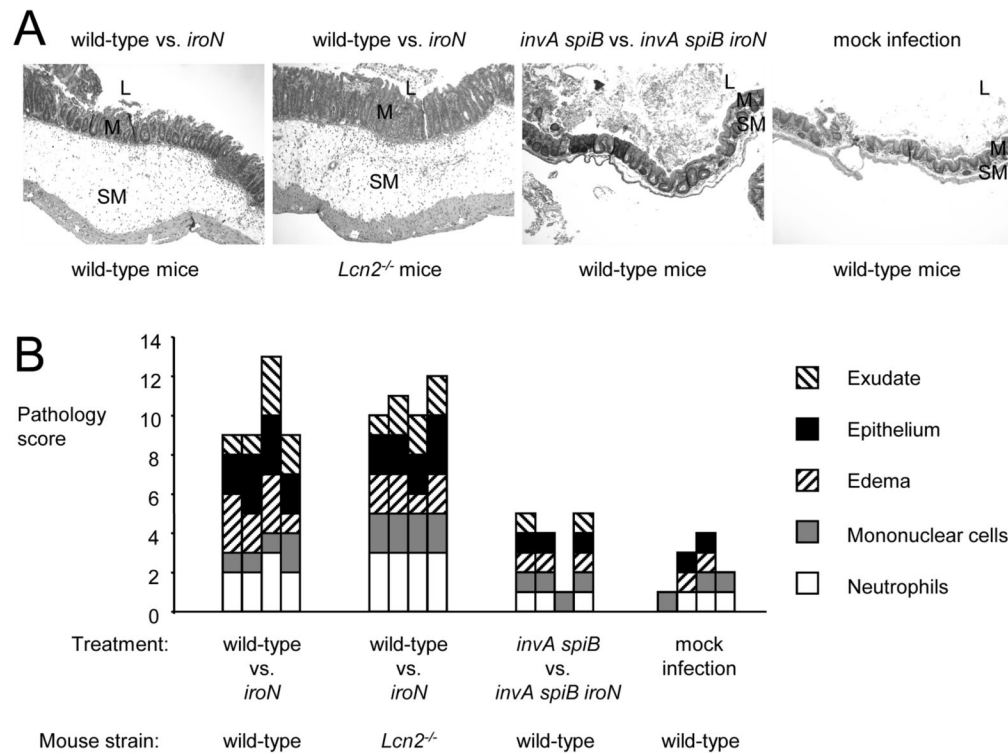


Figure 5. Inflammatory responses elicited in the cecum of streptomycin-pretreated mice 48 hours after *S. Typhimurium* infection assessed by measuring cytokine transcription. Transcript levels of *Il17a* (A), *Il22* (B), *Kc* (C) and *Tnfa* (D) in wild type mice (wt, gray bars) or lipocalin-2 deficient mice (*Lcn2*^{-/-}, black bars) 48 hours after competitive infection (wild-type vs. *iroN* or *invA spiB* vs. *invA spiB iroN*) was measured by quantitative real-time PCR. Bars represent fold-changes (geometric means) in mRNA levels compared to a group of mock-infected wild type mice \pm standard error.

**Figure 6.**

Histopathology of the cecum from mock-infected mice, or mice infected with a mixture of the indicated *S. Typhimurium* strains. (A) Histopathological appearance of the cecum from representative animals in each group. All images were taken from hematoxylin and eosin stained cecal sections at the same magnification (100x). Note marked edema in the submucosa (SM) of mice infected with *S. Typhimurium* wild-type vs. *iroN* mutant. M, mucosa; L, lumen. (B) Pathology score determined by blinded examination of cecal sections under high power magnification. Each bar represents an individual animal.

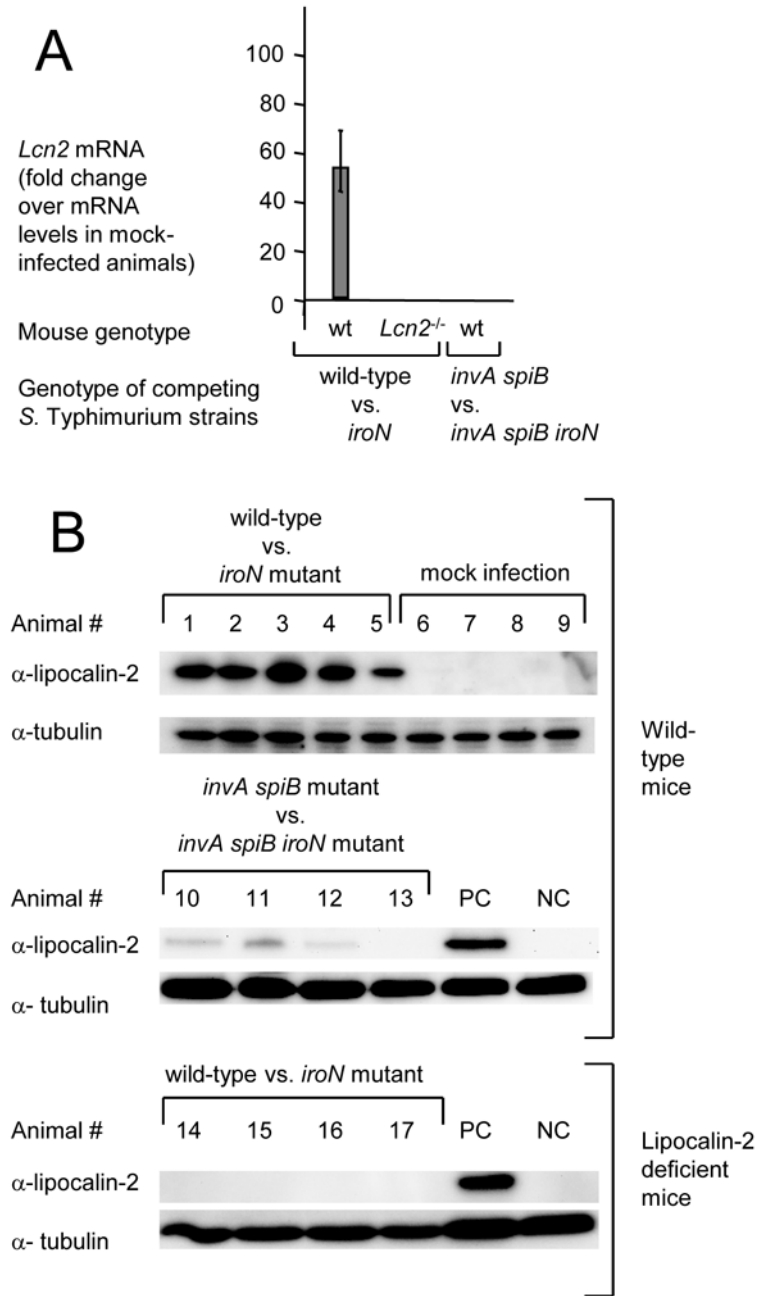


Figure 7. Lipocalin-2 expression detected by quantitative real-time PCR (**A**) or by Western blot (**B**) in the cecal mucosa of streptomycin-pretreated mice 48 hours after *S. Typhimurium* infection. (**A**) Bars represent fold-changes (geometric means) in mRNA levels compared a group of mock-infected wild type mice \pm standard error. (**B**) Lipocalin-2 expression in protein extracts from the cecal mucosa of individual mice was detected with anti-mouse lipocalin-2 antibody (α -lipocalin-2). Detection of tubulin with anti-mouse tubulin antibody (α -tubulin) served as a loading control for each sample. PC, positive control sample from animal #3; NC, negative control sample from animal #8.