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Salmonella Acquires Ferrous Iron from Hemophagocytic Macrophages

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SUMMARY

Bacteria harbor both ferrous and ferric iron transporters. We now report that infection of macrophages and mice with a *Salmonella enterica* Typhimurium strain containing an inactivated *feoB*-encoded ferrous iron transporter results in increased bacterial replication, compared to infection with wild-type. Inactivation of other cation transporters, SitABCD or MntH, did not increase bacterial replication. The *feoB* mutant strain does not have an intrinsically faster growth rate. Instead, increased replication correlated with increased expression in macrophages of the *fepB*-encoded bacterial ferric iron transporter and also required siderophores, which capture ferric iron. Co-infection of mice with wild type and a *feoB* mutant strain yielded a different outcome: FeoB is clearly required for tissue colonization. In co-infected primary mouse macrophages, FeoB is required for *S.* Typhimurium replication if the macrophages were IFNγ treated and contain phagocytosed erythrocytes, a model for hemophagocytosis. Hemophagocytes are macrophages that have engulfed erythrocytes and/or leukocytes and can harbor *Salmonella* in mice. These observations suggest that *Salmonella* acquires ferrous iron from hemophagocytic macrophages.

Keywords

Salmonella; macrophages; iron; hemophagocytosis; bacterial pathogenesis; host-pathogen interaction

INTRODUCTION

Microbial pathogens encode not only genes required for virulence, but also genes that limit virulence, so-called anti-virulence factors. Genes that limit virulence reduce pathogen growth in host tissues or decrease the dose required for host death (Baek CH, 2009; Foreman-Wykert AK, 2003; Gal-Mor O, 2008). In laboratory screens, genes that reduce virulence have been identified based on hyper-virulence phenotypes in loss-of-function mutant strains upon single-infection of mice or macrophages (Ho TD, 2001). Indicators of hyper-virulence include colonization advantages, decreased survival time of the host, and reduced lethal or infectious dose (Baek CH, 2009; Foreman-Wykert AK, 2003; Gal-Mor O,

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2008). While multiple examples of pathogen loci that limit virulence have been described (Baek CH, 2009; Gal-Mor O, 2008; Pilonieta MC, 2012), it remains largely unknown as to how they limit virulence and why mutations in these loci do not come to dominate the population.

The Gram-negative bacterium *Salmonella enterica* acts as a stealth pathogen to colonize tissues in human typhoid fever and in murine models of typhoid fever. During systemic infection, *Salmonella* evades humoral immunity by living within host cells (Tsolis RM, 2008). One factor that may make it difficult for the host to respond to *Salmonella* is that bacterial replication is limited *in vivo* within macrophages. In the mouse spleen and liver, individual macrophages typically harbor only four to five bacterial rods (Nix, 2007; Sheppard M, 2003). Low bacterial numbers likely reflect a combination of bacterial killing by cellular innate immunity and limited bacterial replication. Bacterial replication is restricted by antimicrobial peptides (Rosenberger CM, 2002) and reactive nitrogen species (Vazquez-Torres A and Fang, 2001), which have the potential to cause bacterial DNA damage (Buchmeier NA, 1993; Buchmeier NA, 1995; Craig M, 2009; Rosenberger CM, 2002; Suvarnapunya AE, 2003). It is unclear why, in the face of DNA damaging agents, the bacterial genome within a host appears to be fairly stable, and hyper-virulent clones do not typically overrun an infection or epidemic (Clairmont C, 2000; Holt KE, 2008).

Another factor that may allow *Salmonella* to evade killing is that the bacteria can apparently reside in different kinds of host cells. In a mouse model of chronic infection of the gall bladder, *S.* Typhimurium resides within gall bladder epithelial cells and macrophages, a professional phagocyte that normally kills bacteria (Gonzalez-Escobedo, 2013). In mice in which bacteria persist within the mesenteric lymph nodes, spleen and liver, *S.* Typhimurium resides within subsets of macrophages, including hemophagocytes and alternatively activated macrophages (Eisele NA, 2013; Nix, 2007). Hemophagocytes are macrophages that accumulate during acute inflammation and engulf apparently non-senescent red and white blood cells (Fisman, 2000; McCoy MW, 2012; Silva-Herzog E, 2008). Why *S.* Typhimurium resides within hemophagocytes is unclear.

Here we establish that a highly conserved iron importer, FeoB, limits *S.* Typhimurium replication in macrophages and tissues. However, the *feoB* mutant strain is severely attenuated upon co-infection of mice with wild-type bacteria. We observe a requirement for *feoB* upon co-infection particularly of IFNγ-activated macrophages that have engulfed erythrocytes, indicating a need for FeoB iron uptake in hemophagocytes.

RESULTS

FeoB limits S. Typhimurium replication in macrophages

The FeoAB system transports ferrous iron from the periplasm to the cytosol (Kammler M, 1993). A *feoB* deletion mutant strain was constructed by replacing the *feoB* open reading frame with a kanamycin resistance cassette. We confirmed that there are no significant growth differences between the wild-type and Δ*feoB* strains in nutrient rich media (LB) or nutrient poor media (M9) at 37°C (Figure 1 A and 1B) (Boyer E, 2002). In addition, iron chelation with 2’2 dipyridyl limited growth of the Δ*feoB* strain (OD_{600} = 0.46 after 18 hours

of growth, compared to OD_{600} = 0.85 without chelation), as previously reported (Tsolis RM, 1996). Macrophages are a key cell type in which *S.* Typhimurium resides *in vivo* (Monack, 2004), and yet a *feoB* mutant strain had no apparent survival defect in macrophage-like RAW264.7 cells (Boyer E, 2002). However, RAW264.7 cells are especially permissive for *S.* Typhimurium replication because they express an unstable mutant form of Nramp1/Slc11a1, a divalent cation transporter with pleiotropic effects on innate immunity (Barton CH, 1995; Canonne-Hergaux F, 1999; Govoni G, 1998). To establish whether a *feoB* mutant has a survival defect in Nramp1+ macrophages, bone marrow-derived macrophages (BMDMs) isolated from Sv129S6 $Nramp1^{+/+}$ mice were examined. We infected resting BMDMs with wild type or a *feoB* mutant *S.* Typhimurium and performed gentamicin protection assays. Unexpectedly, strains lacking *feoB* replicated at a higher rate than wild type (Figure 1C). Classically (IFNγ and LPS) activated BMDMs also allowed the *feoB* mutant to replicate more than wild type (Figure 1D). A strain in which the deleted *feoB* locus was restored with a wild-type *feoB* gene on the chromosome replicated only as well as wild type (Figure 1D). RAW264.7 cells carrying a wild-type $Nramp1^{G169}$ transgene showed similar results (Van Zandt KE, 2008) (Figure S1). A control strain in which type three secretion systems (T3SS) 1 and 2 were inactivated in a *feoB* mutant background (Δ*feoB*, Δ*invA*, Δ*spiC)* was unable to replicate in macrophages, indicating that, as expected, intracellular replication requires T3SS-2 (data not shown) (Cirillo, 1998; Hensel, 2000). These data collectively show that FeoB limits *S.* Typhimurium replication in cultured macrophages.

FeoB limits S. Typhimurium tissue colonization in the spleen and liver of mice

We next examined tissue colonization by the Δ*feoB* mutant strain in Sv129S6 $Nramp1^{+/+}$ mice orogastrically inoculated with a wild type or mutant strain. Feces were collected and plated for CFU at one and three days post-infection to determine the degree of intestinal colonization for each strain. At one-day post infection, more Δ*feoB* bacteria were recovered from fecal pellets, but by three days there was no difference (Figure 2A). At two weeks post-infection, mice were sacrificed and the spleen, liver, mesenteric lymph nodes, and Peyer's patches were removed, homogenized and plated to determine bacterial tissue loads. The *feoB* mutant colonized the spleen and the liver to significantly higher levels than the wild-type strain (Figure 2B). These results are consistent with the increased replication of the *feoB* mutant in Nramp1+ macrophages and suggest that FeoB limits tissue colonization in mice.

The divalent cation transporters SitABCD and MntH do not limit tissue or macrophage colonization

While FeoB appears to be largely dedicated to transporting ferrous iron (Fe^{2+}), *S.* Typhimurium also encodes two divalent cation transporters with broader specificity (Kammler M, 1993; Zhou D, 1999). MntH and SitABCD have high affinity for manganese (Mn^{2+}) and 10-100-fold lower affinity for Fe^{2+} (Kehres, 2000; Makui H, 2000). To establish whether SitABCD or MntH influence the course of infection, mice were orogastrically inoculated with individual wild-type, Δ*sitA* or Δ*mntH* strains. The *sitA* deletion strain was recovered from fecal pellets at slightly lower levels at one-day post-infection compared to wild-type, but within three days all strains were recovered equivalently (Figure 3A). At two

weeks post-infection, colonization of both mutant strains was indistinguishable from that of wild type in the spleen, liver, mesenteric lymph nodes and Peyer's patches (Figure 3B, C). In the cecum, fewer Δ*mntH* mutant bacteria were recovered compared to wild type, indicating this transporter may be required specifically for intestinal colonization. In RAW264.7 Nramp1+ macrophage-like cells, Δ*mntH* and Δ*sitA* mutants replicated at similar rates as the wild-type strain (Figure 3D). These data show that neither the SitABCD nor MntH cation transporters limit bacterial replication *in vivo* in the Sv129S6 Nramp1$^{+/+}$ orogastric infection model.

FeoB reduces expression of the Fep ferric iron transporter in macrophages

In the presence of ferrous iron, the Fur transcription factor binds DNA and represses genes encoding iron uptake proteins. Therefore, disruption of the *feoB* encoded ferrous iron importer may increase expression of ferric iron uptake genes (Hantke, 1981, 1987; Pecqueur L, 2006; Tsolis RM, 1995). One such Fur-regulated gene is *fepB*, which encodes a key periplasmic protein of a ferric iron uptake system required for *S.* Typhimurium survival in macrophages and mice (Crouch ML, 2008; Nagy TA, 2013). To determine whether a *feoB* mutant strain has increased *fepB* expression, total RNA was isolated from macrophages infected with wild-type or Δ*feoB* strains. RTPCR revealed that *fepB* transcripts were approximately 8-fold more abundant in Δ*feoB* mutant bacteria compared to wild-type (Figure 4A), indicating that in macrophages *S.* Typhimurium increases *fepB* expression in the absence of *feoB*.

Siderophores are required for increased colonization by the feoB mutant strain

Siderophores capture ferric iron and are transported through the outer membrane for delivery to FepB. IroN and FepA are siderophore transporters required for virulence in *S.* Typhimurium (Crouch ML, 2008; Gorbacheva VY, 2001; Nagy TA, 2013; Rabsch W, 2003; Rabsch W, 1999; Williams PH, 2006). To establish whether increased replication of strains lacking *feoB* requires siderophore capture through *iroN* and *fepA*, we constructed a triple mutant Δ*feoB*, Δ*iroN*, Δ*fepA* strain. The triple mutant and wild-type control strains replicated similarly in macrophages and colonized tissues to similar levels in mice (Figure 4B, C). Thus, increased replication of the Δ*feoB* mutant strain depends upon the presence of siderophores. We also compared the ability of the wild type, Δ*iroN*, Δ*fepA* double mutant (Nagy TA, 2013), and Δ*iroN*, Δ*fepA*, Δ*feoB* triple mutant strains to colonize the spleen and liver (Figure 4D), the two tissues in which Δ*feoB* mutant strains hyper-replicate (Figure 2B). The colonization defect of the double mutant (Nagy 2013) is suppressed by the loss of *feoB*. One possibility is that this suppression is mediated by the induction of Fur-repressed iron acquisition genes.

In mixed-infections of mice, FeoB is required for S. Typhimurium replication

We next established whether FeoB limits bacterial replication when the mutant strain is competing with the wild-type parent strain within an animal. We inoculated Sv129S6 Nramp1$^{+/+}$ mice with equivalent numbers (1×10^9 CFU) of differentially marked wild-type and mutant bacteria in PBS. At one day post-infection, wild type and the *feoB* mutant strain were recovered from fecal pellets at similar levels. By three days post-infection, wild type

outcompeted Δ*feoB* in fecal pellets by 10-fold (Figure 5A). Tissues were harvested, serially diluted and plated to enumerate CFU two weeks post-infection. Fewer than 100 wild-type and mutant colonies were recovered from the cecum of all animals (data not shown). In the Peyer's patches, mesenteric lymph nodes, spleen, and liver wild-type bacteria outcompeted the Δ*feoB* mutant by 10,000-fold (Figure 5B). In a different strain background, C57Bl6 mice containing an Nramp1^{G169} transgene and therefore resistant to *S.* Typhimurium, wild-type bacteria also outcompeted the Δ*feoB* mutant strain (Figure 5C), consistent with previous observations (Fritsche G, 2012). The cation transporters MntH and SitABCD were also confirmed to be important in mixed-infections of mice (Figures 5D and 5E) (Janakiraman A, 2000). Thus, the *feoB* transport system is strongly required for tissue colonization in competition with wild-type bacteria.

FeoB limits S. Typhimurium replication in macrophages upon mixed-infection

The difference in tissue colonization of the Δ*feoB* strain compared to wild type in single (+10-fold) versus mixed (-10,000-fold) infection of mice was striking. To establish whether this difference may reflect macrophage responses to infection, we examined bacterial replication upon co-infection of macrophages. RAW264.7 Nramp1+ macrophages that were resting, classically activated (treated with IFNγ and LPS), or alternatively activated (treated with IL-4), were inoculated with equivalent numbers of both strains. As in single-infections, the *feoB* mutant outcompeted wildtype (Figure 6A-C), indicating that co-infection of RAW264.7 Nramp1+ macrophages does not model co-infection in Nramp1+ mice with regard to FeoB.

Since RAW264.7 Nramp1+ macrophages allow for *S.* Typhimurium replication, whereas BMDMs kill the bacteria (Figure S2), we next examined replication of the *feoB* mutant strain in competition with wild type in BMDMs from Sv129S6 (Nramp1$^{+/+}$) mice. BMDMs remained resting, or were treated with IFNγ, both LPS and IFNγ, or IL-4. Under these conditions, the Δ*feoB* mutant strain had a small but insignificant advantage over the wild-type strain within 24 hours of infection in resting and IL-4 treated macrophages (Figure 7A). As expected, treatment with IL-4 increased the percentage of BMDMs that express CD301, a marker of alternative activation (Figure 7C). These data indicate that mixed-infection of BMDMs does not recapitulate the strong requirement for *feoB* observed in mixed-infections of mice.

FeoB is required for replication in hemophagocytes upon mixed-infection

Since *S.* Typhimurium can reside within hemophagocytes in mice (Nix, 2007), to model hemophagocytosis we inoculated BMDMs with freshly isolated erythrocytes. Prior to adding erythrocytes, the BMDMs were resting or activated. IFNγ treated or IFNγ and LPS treated BMDMs allowed wild-type bacteria to outcompete the Δ*feoB* mutant (Figure 7B), similar to results obtained in mice (Figure 5B,C). These data suggest that macrophages incubated with erythrocytes more accurately model the mouse response to infection with respect to *feoB.*

We next determined whether macrophage engulfment of erythrocytes contributed to the ability of the wild-type strain to outcompete the *feoB* mutant strain in individual BMDMs. Hemophagocytes (HMs) and non-hemophagocytes (NHMs) were distinguished by flow

cytometry; extracellular erythrocytes were removed by lysis and macrophages containing erythrocytes (HMs) were identified with an antibody to an erythrocyte epitope, TER-119. IFNγ activation resulted in the accumulation of more HMs than did treatment with media alone (Figure 8A). HMs and NHMs were examined for the presence of wild-type (RFP signal) and/or Δ*feoB* (GFP signal) bacteria (Figure 8B). Few macrophages under any conditions supported the *feoB* mutant strain (gray bars) within 18 hour of infection. In contrast, more HMs than NHMs, whether resting or activated, harbored the wild-type strain (black bars) (Figure 8C). These data indicate that macrophages containing erythrocytes provide wild type but not *feoB* mutant bacteria with a survival niche.

Median fluorescence intensity (MFI) can serve as a proxy for the relative number of bacteria in a given cell population (Figure S3). In resting macrophages (Figure 8D, left) the *feoB* mutant strain outcompeted the wild-type strain, consistent with expression of Fur-repressed genes in the *feoB* mutant in macrophages (Figure 4). In activated macrophages, the *feoB* mutant replicated poorly under all conditions compared to wild type, including in macrophages in which the *feoB* mutant strain was the only bacterial strain detected (Figure 8D, right). This result appears to contradict previous observations of increased *feoB* mutant replication in activated primary macrophages (Figure 1D). However, the activated NHMs in the current experiment were exposed to erythrocytes and therefore hemoglobin and heme, for which macrophages express receptors (Fabriek BO, 2005; Hvidberg V, 2005). In activated NHMs incubated with erythrocytes, the *feoB* mutant strain may obtain just enough iron to maintain Fur-mediated repression of iron acquisition genes, but not enough iron to replicate. In activated HMs containing both bacterial strains, the wild-type strain replicated to the highest levels (Figure 8D, right). These results support the observation that IFNγ stimulates macrophage export of iron, thereby reducing pathogen access to iron within the macrophage (Nairz M, 2008). The presence of erythrocytes in IFNγ-activated macrophages appears to compensate for iron export by increasing bacterial access to ferrous iron and thus replication. Without the *feoB* gene, *S.* Typhimurium cannot take full advantage of erythrocytes in macrophages, suggesting that wild-type *S.* Typhimurium requires FeoB to access ferrous iron within hemophagocytes.

DISCUSSION

Our results show that *feoB* is required for *S.* Typhimurium replication during co-infection of hemophagocytic macrophages. However, *S.* Typhimurium lacking *feoB* indeed has enhanced virulence compared to wild type upon single-infection of Sv129S6 (Nramp1$^{+/+}$) mice and macrophages. This observation may reflect increased expression of iron acquisition genes in the Δ*feoB* mutant strain upon macrophage infection. Under low iron conditions, induction of genes repressed by Fur in the presence of iron, including *fepB, iroN* and *fepA,* occurs in *E. coli* and *S. enterica* (Bäumler AJ, 1998; Brickman TJ, 1990; Tsolis RM, 1995).

Enhanced ferric iron uptake in response to ferrous iron limitation appears to be a common pathogen strategy. The alpha-proteobacterium *Brucella abortus* also lives within macrophage vesicles and causes chronic infection. *B. abortus* requires a ferrous iron uptake system for virulence in mice (Elhassanny AE, 2013) but also captures heme as an iron source (Paulley JT, 2007) and encodes at least one hemeoxygenase, BhuQ (Ojeda JF, 2012).

Under low iron conditions, *B. abortus* increases expression of a siderophore-encoding gene, *dhbC*, in a *bhuQ* mutant strain (Ojeda JF, 2012). Similarly, in Nramp1$^{+/+}$ mice and macrophages, *S. enterica feoB* mutant strains may compensate for iron starvation by inducing Fur-dependent iron acquisition genes to support enhanced bacterial replication.

Mixed-infection experiments of mice with the *feoB* mutant yielded very different results from single-infection experiments. This difference may reflect that the host has a more vigorous immune response to *S.* Typhimurium that expresses FeoB. In *Yersinia pseudotuberculosis*, another gamma-proteobacterium, YopE and YopH are virulence determinants secreted into host cells (Trosky JE, 2008). Infection of mice with either a *yopE* or *yopH* mutant strain yields tissue colonization comparable to infection with the parent strain, but both mutant strains are outcompeted by the wild-type strain in mixed-infections. A series of elegant experiments demonstrated that wild-type *Y. pseudotuberculosis* induces more robust inflammatory responses than *yopE* or *yopH* mutant strains, and that growth of a *yopE* mutant strain is inhibited by inflammation (Logsdon LK, 2006). With regard to *S.* Typhimurium, the presence of a wild-type strain expressing FeoB may stimulate in mice and in IFNγ-activated hemophagocytes an immune response that that prevents replication of a co-infected strain lacking FeoB.

A second explanation for the discrepancy of the *feoB* mutant phenotype in mixed and single infections is based on the iron transport role of FeoB. Erythrocytes are degraded within macrophage vesicles, followed by heme transport into the cytosol for catalysis by hemeoxygenase-1 and release of ferrous iron (Delaby C, 2012). Perhaps during mixed-infection wild-type *S.* Typhimurium captures heme-derived iron with FeoB and thereby outcompetes the *feoB* mutant strain before the latter can induce ferric iron acquisition genes. Upon single-infection, the *feoB* mutant appears to be sufficiently iron starved and has enough time to reverse Fur-mediated repression of ferric iron uptake. Macrophage treatment with IFNγ results in ferrous iron export from the cell and thereby restricts *S.* Typhimurium replication (Nairz M, 2008). However, hemophagocytosis appears to counter the effect of IFNγ, probably based on ferrous iron release from heme followed by FeoB-dependent capture of ferrous iron by *S.* Typhimurium. In contrast, in macrophages without erythrocytes *S.* Typhimurium must import ferric iron via siderophores and FepB (Nagy TA, 2013). Altogether, the data suggest that hemophagocytes more faithfully model the host environment than other kinds of macrophages by providing *S.* Typhimurium with iron.

In conclusion, despite appearing to be an anti-virulence determinant in single-infection experiments, FeoB is clearly required for virulence during mixed infection. Mixed infection is the more realistic context with respect to pathogen evolution in the host because genetic bacterial variants that arise *in vivo* must compete with much larger numbers of the wild-type parent strain. If a spontaneous loss-of-function mutation in *feoB* were to arise during infection, it would not become fixed within the population because it could not survive in the presence of wild-type bacteria. Our study also indicates that FeoB and thus ferrous iron transport is important particularly in hemophagocytes, indicating that *S.* Typhimurium exploits these macrophages for access to iron.

EXPERIMENTAL PROCEDURES

Construction of bacterial strains and growth conditions

Salmonella enterica serovar Typhimurium wild-type strain SL1344 (Merritt FF, 1984) and mutant derivatives were grown overnight at 37°C with aeration prior to infections. Antibiotics were used at the following concentrations: streptomycin, 30 μg/mL and kanamycin, 30 μg/mL.

Strains with deletions of iron transporters were made following the Wanner insertion method. Briefly, strains with marked deletions of *mntH, sitA* or *feoB* were constructed in the wild-type background by recombination of the PCR product derived from primers specific for the region flanking the corresponding gene and the kanamycin resistance marker on plasmid pKD4 (Datsenko KA and Wanner, 2000). The following primers were used: *mntH* fwd: 5'-ATGAAACATAGCAAAGGCTATGTTTTTGAGGCAAAAGgtgtaggctggagctgctt-3' *mntH* rev: 5'-ACGCCCACGCATCGGGCCTGCTATCTTTCTATCTcatatgaatatcctccttag-3'; *feoB* fwd: 5'-GTAAAAAGGATTTGGCGTTAATAGAAGTGGAAGCGGgtgtaggctggagctgcttc-3' and *feoB* rev: GAACCTGTATCAATGAAGCCATTTTTTACATCCCcatatgaatatcctccttag- 3'; *sitA* fwd: 5'-TCGATGATTAATTAACCACATTGTTGCGAGGGATACTgtgtaggctggagctgcttc-3' and *sitA* rev: 5'-ACCGTGACTTGATCAACGGTAATCGCAGATTGACcatatgaatatcctccttag-3'. Lower case letters indicate the primers designed previously (Datsenko KA and Wanner, 2000). Kanamycin insertions were confirmed by PCR. The Δ*feoB,* Δ*invA,* Δ*spiC* mutant strain was created by P22 phage transduction of Δ*feoB*-kanamycin resistance into to an unmarked Δ*invA,* Δ*spiC* double mutant strain (Silva-Herzog, 2010).

Growth curves

Overnight cultures were diluted to an OD_{600} of 0.01 in 200 μL of Luria-Bertani (LB) medium or M9 minimal medium (M9). Bacteria were grown in 96-well plates shaking in a Synergy2 plate reader (BioTek) at 37°C for 17 hours. The OD_{600} was recorded at 20-minute intervals.

Mouse infections

Research protocols were approved by the University of Colorado Institutional Committees for Biosafety and for Animal Care and Use. For mixed-infection studies, 7-week-old male and female 129SvEvTac ($Nramp1^{G169/G169}$) mice (Taconic Laboratories) bred in-house were fasted for two hours prior to orogastric inoculation with 1×10^9 wild-type and 1×10^9 mutant bacteria, as verified by plating for CFU on selective LB agar, in 100uL of PBS. C57/BL6 $Nramp1^{G169D}$ mice carrying an $Nramp1^{G169}$ (SLC11a1) transgene (Govoni G, 1998; Kuhn DE, 2001) were obtained from Dr. F. Fang (Brown DE, 2013).

For single-infection experiments, mice were fasted for two hours to allow gastric contents to clear. Mice were inoculated orogastrically with 1×10^9 bacteria as verified by plating for

CFU on selective LB agar. At one and three days post-infection, at least two fecal pellets were collected from each mouse in 1 mL of PBS, vortexed until homogeneous, diluted and plated on selective media. Two weeks after inoculation, tissues (spleen, liver, mesenteric lymph nodes, Peyer's patches, and cecum) were collected in 1 mL PBS, homogenized with a TissueMiser (Fisher Scientific) and diluted in PBS for plating on selective LB agar plates. CFU were enumerated and the competitive indexes (CIs) were calculated as follows: ($CFU_{wild\text{-}type}/CFU_{mutant}$) output / ($CFU_{wild\text{-}type}/CFU_{mutant}$) input. If fewer than 100 bacteria total or from each strain (for mixed-infections) were recovered, then CIs were not calculated.

Cell culture gentamicin protection assays

Primary macrophages were isolated as previously described (Nix, 2007). Briefly, marrow was flushed from the femurs and tibias of 2- to 4-month-old 129SvEvTac mice (Taconic Laboratories). Cells were resuspended in Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with fetal bovine serum (10%), l-glutamine (2 mM), sodium pyruvate (1 mM), beta-mercaptoethanol (50 μM), HEPES (10 mM), and penicillin-streptomycin (50 IU/mL penicillin and 50 μg/mL streptomycin). Cells were overlaid onto an equal volume of Histopaque-1083 (Sigma-Aldrich, St. Louis, MO) and centrifuged at 1500rpm for 10 minutes. Monocytes at the interface were harvested and incubated for six days at 37°C in 5% CO_2 in supplemented DMEM that also contained 30% m-CSF conditioned medium to promote monocyte differentiation into macrophages.

Bone marrow-derived macrophages and RAW264.7 $Nramp1^{+/+}$ cells were seeded at 1.5×10^5 cells per well in poly-L-lysine-coated 24-well tissue culture plates. Cells were classically activated with 20 ng/mL lipopolysaccharide (S. *enterica* Typhimurium LPS; Sigma-Aldrich) and 20 U/mL IFNγ (PeproTech) for 18 hours and alternatively M2 activated with 20 ng/mL murine IL-4 (PeproTech) for 24 hours. Where indicated, freshly isolated murine erythrocytes were added at a 10:1 ratio for 1 hour. Bacteria were added to macrophages at a multiplicity of infection of 10. After 30 minutes, cells were washed and incubated for 1.5 hours at 37°C in fresh media supplemented with gentamicin (100 μg/mL) to kill extracellular bacteria. Media was then exchanged for media supplemented with gentamicin (10 μg/mL) to prevent extracellular bacterial growth. At 2, 18 and 24 hours, wells were washed twice with pre-warmed PBS, incubated with 1% Triton X-100 for 5 minutes, lysed, and serial dilutions plated for colony-forming units.

Real-time reverse transcriptase PCR

1.5×10^5 macrophages were infected with wild-type *Salmonella* or Δ*feoB* mutants or medium alone for two hours as described in *Cell culture gentamicin protection assays.* Macrophages were scraped and collected into 1.5 mL eppendorf tubes and spun at 16,000rpm for 10 minutes to lyse macrophages. The supernatant from lysed macrophages was removed and RNA was prepared from bacterial pellets using RNAeasy kit (Qiagen) following the manufacturer's instructions after treatment with lysozyme. Reverse transcriptase-PCR was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems), which was followed by quantitative PCR using SYBR green (Applied Biosystems) and the Eppendorf Mastercycler ep *realplex*^{2} S (Eppendorf) qPCR

machine. Relative differences between bacteria were calculated based on values for the *fepB* (fwd primer: 5'ggcactaaggcttgcatctc3'; rev primer: 5'tcaacgtcacgctggtagag3') gene normalized to values of the 16s (universal fwd primer: 5'gtgccagcmgccgcggtaa3'; rev primer: 5'gacgggcggtgtgtrca3') gene.

Flow cytometry

Resting or IFNγ-activated macrophages seeded in a 6-well plate were incubated with freshly isolated murine erythrocytes at a 10:1 ratio for one hour prior to co-infection with wild-type SL1344 containing a plasmid expressing dsRed and the Δ*feoB* strain containing a plasmid expressing eGFP. At 18 hours post-infection, cells were washed with cold PBS and erythrocytes were lysed with potassium bicarbonate ACK lysis buffer (Bossuyt X, 1997) and harvested by gentle scraping. Cells from each condition were equally distributed into 96-well plates and resuspended in FACS staining buffer (PBS plus 1% fetal bovine serum (FBS), 0.02% azide) containing anti-mouse CD16/32 (eBioscience) to block Fc receptors. Cells were then fixed on ice in 1% paraformaldehyde–1% sucrose, permeabilized in staining buffer with 0.1% saponin, and then incubated in staining buffer containing 0.5 μg/sample anti-mouse Ter-119-phycoerythrin (PE)-Cy7 (eBioscience). Fluorescently labeled cells were quantified with a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA) and analyzed with appropriate compensation using FlowJo software (Tree Star, Inc.). Any remaining intact extracellular erythrocytes were removed by gating, as erythrocytes have less SSC and FSC relative to macrophages.

Statistics

p values were calculated with GraphPad Prism 5 (GraphPad Software Inc.) and considered significant if $p < 0.05$. For nonparametric data, Wilcoxon signed-rank or Mann-Whitney tests were used. Otherwise, student's t-test or ANOVA were used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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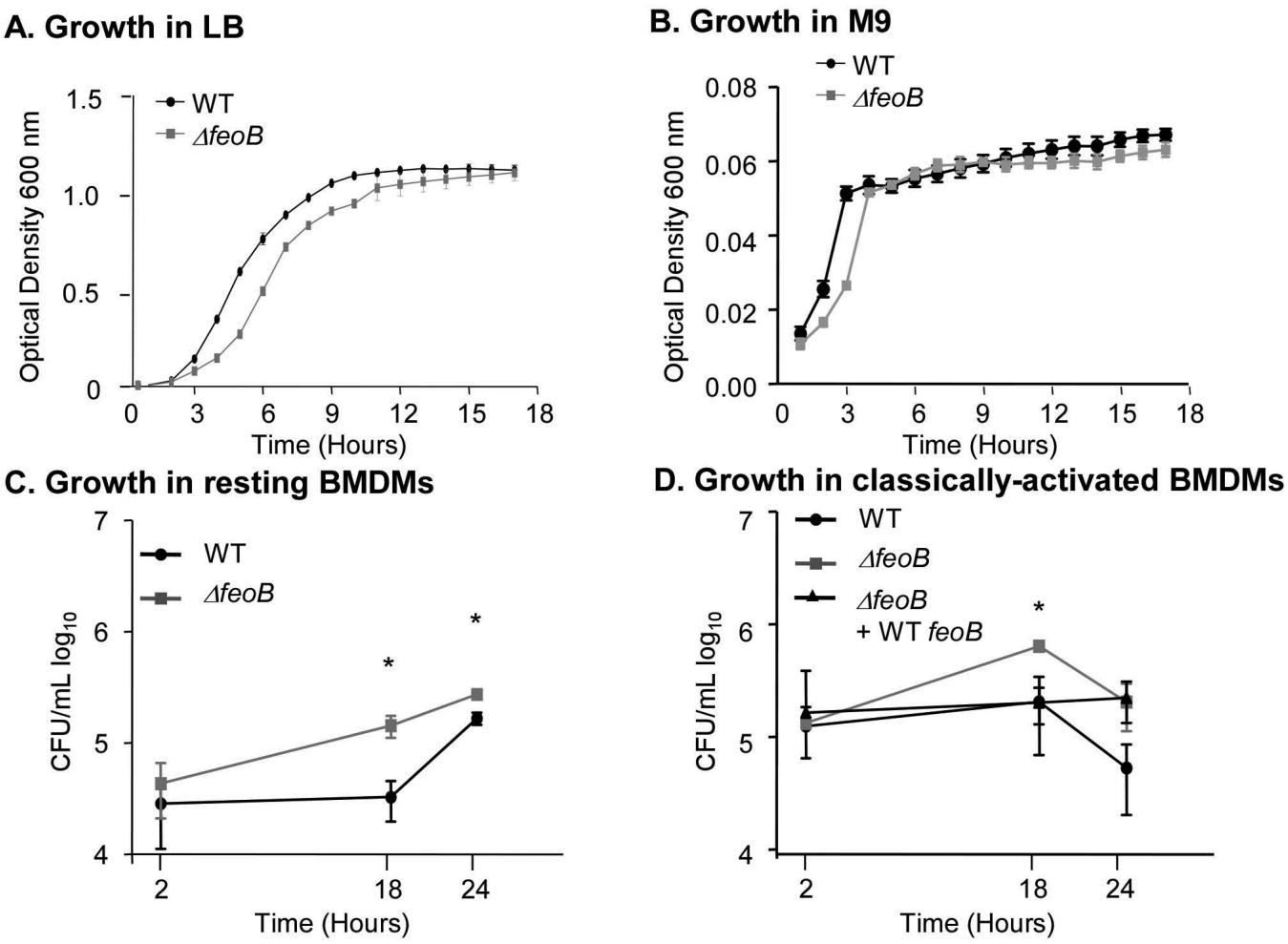

Figure 1. FeoB limits *S*. Typhimurium replication in macrophages

Wild-type (WT) or Δ*feoB* mutant strains were grown in LB (A) or M9 (B) minimal medium and optical density at 600 nm was monitored for 17 hours. Error bars are SD, n ≥ 3 experiments. Bone marrow-derived macrophages (BMDMs) isolated from Sv129S6 ($Nramp1^{+/+}$) mice were resting (C) or classically activated (IFNγ and LPS) (D). Macrophages were inoculated with the strains indicated at an MOI of 10. Mean and SD of representative experiments are shown. *p*-values were determined as described in the methods. $p \leq 0.05$ (*) vs. WT, n ≥ 3 experiments.

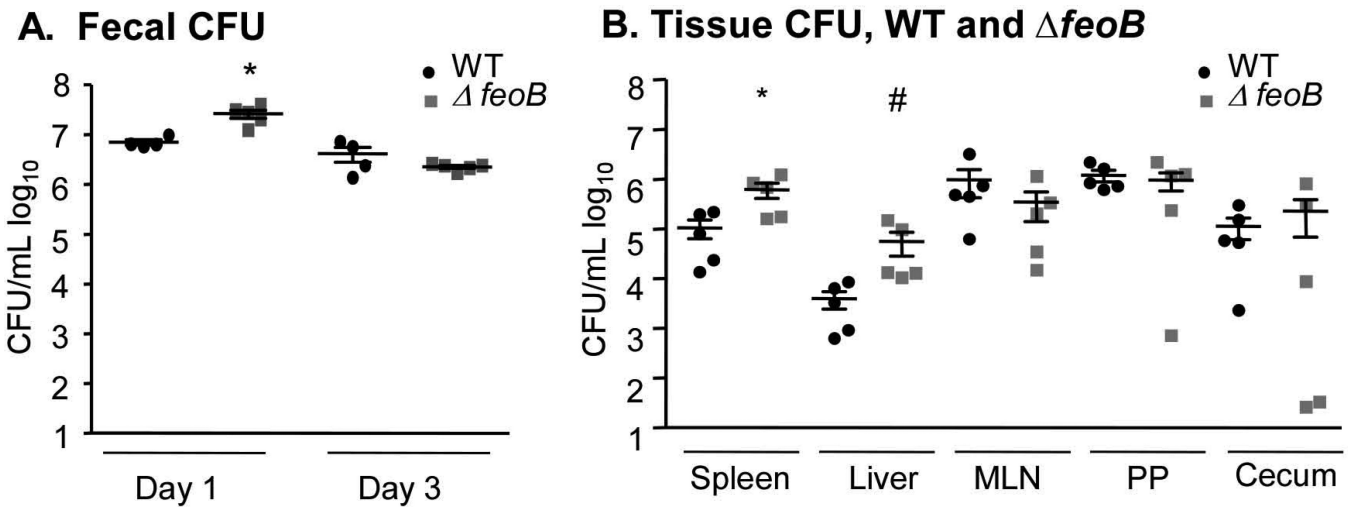

Figure 2. FeoB limits colonization of the spleen and liver in Sv129S6 (Nramp1$^{+/+}$) mice

Mice were orogastrically inoculated with either the WT or a Δ*feoB* mutant strain. Fecal pellets were harvested 1 and 3 days post-infection (A). Tissues were harvested and plated for CFU at 2 weeks post-infection (B). Mean and SEM are shown. *p*-values were determined as described in the methods. $p < 0.05$ (*) vs. WT; $p < 0.01$ (#) vs. WT. Each symbol represents one mouse, n = 5.

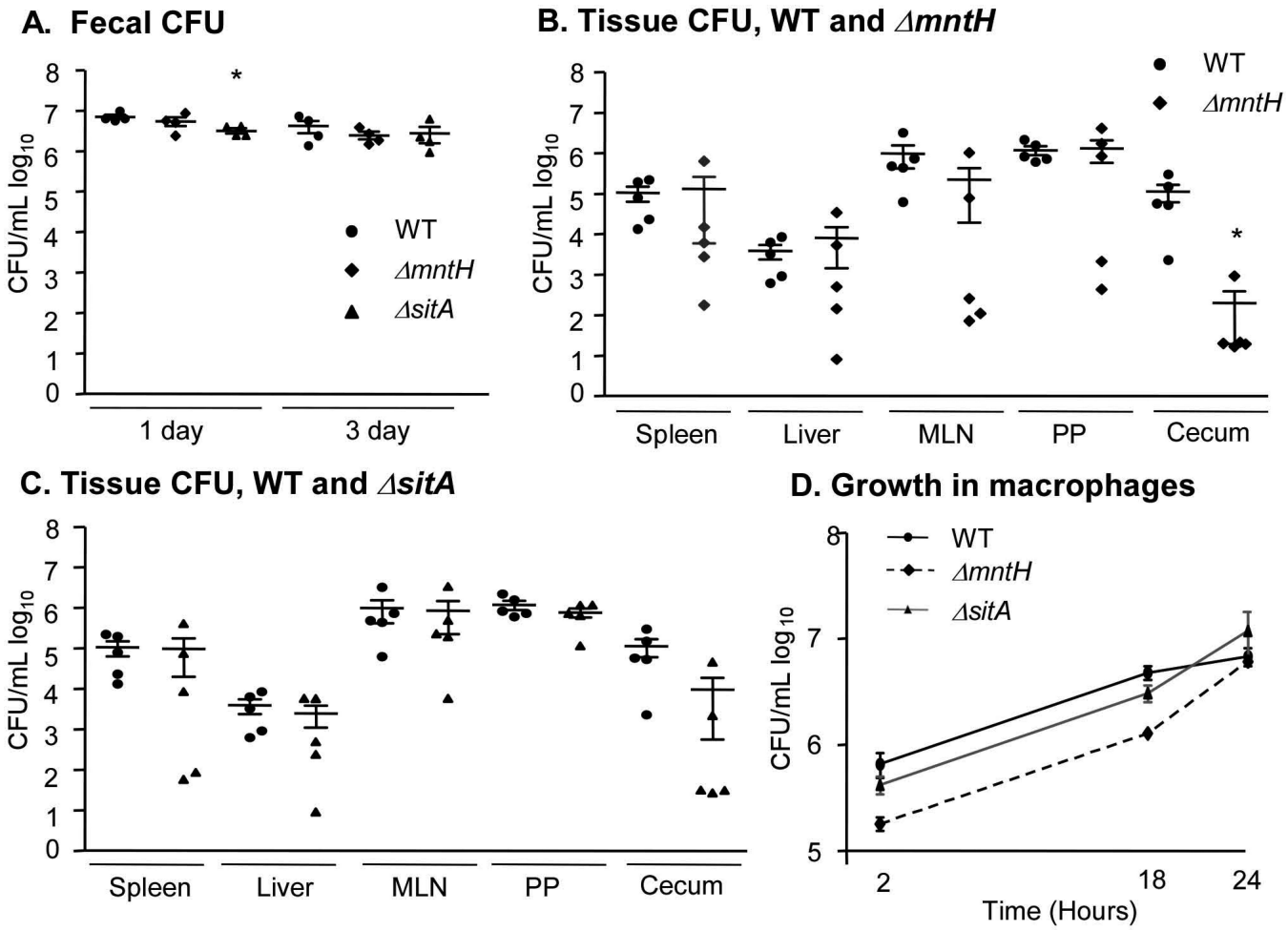

Figure 3. Strains lacking *sitA* or Δ*mntH* do not limit replication in mice or macrophages

Mice were orogastrically inoculated with WT, Δ*sitA* or Δ*mntH* strains. Fecal pellets were harvested at 1 and 3 days post-infection (A). Tissues were harvested and plated for CFU at 2 weeks post-infection (B, C). Mean and SEM are shown. *p*-values were determined as described in the methods. $p < 0.05$ (*) vs. WT. Each symbol represents one mouse, n = 5. D) RAW264.7 Nramp1+ cells were treated with IFNγ and LPS and inoculated with the strains indicated at an MOI of 10. Mean and SD of a representative experiment are shown. n ≥ 3 experiments.

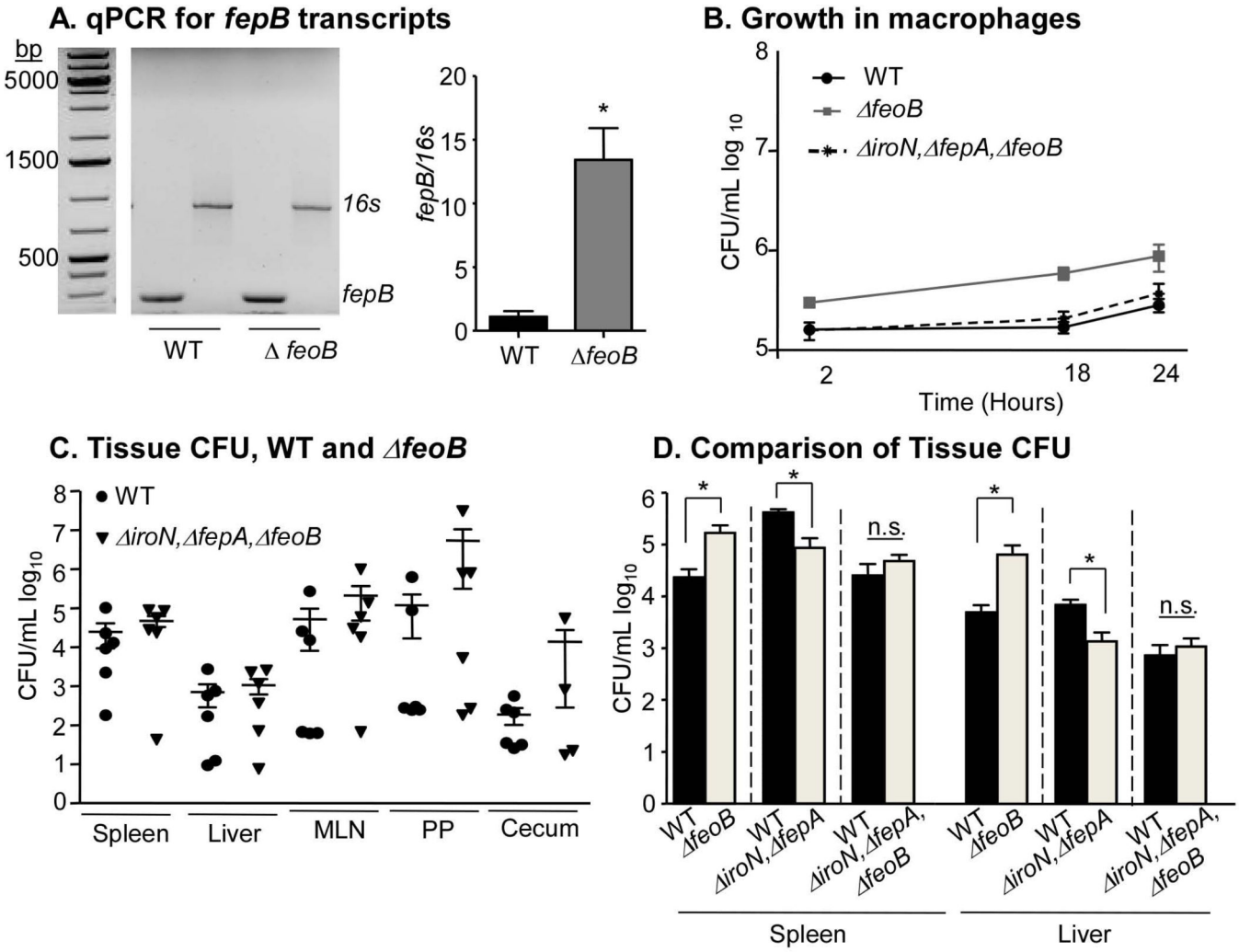

Figure 4. Increased replication in the absence of *feoB* is dependent on ferric iron uptake

A) RAW264.7 Nramp1+ cells were infected with WT or Δ*feoB* mutant strains at an MOI of 10. At 2 hours post-infection, macrophages were lysed and isolated RNA was subjected to real-time RT-PCR. Agarose gel, left, quantification, right. *fepB* amplicon, 245bp and 16s amplicon, 850bp. Mean and SEM are shown. $p < 0.05$ (*), n = 3 experiments. B) IFNγ and LPS activated RAW264.7 Nramp1+ cells were inoculated with the strains indicated at an MOI of 10. Mean and SD of a representative experiment is shown. $p < 0.05$ (*), n ≥ 3 experiments. C) Mice were orogastrically inoculated with WT or Δ*iroN*, Δ*fepA*, Δ*feoB* strains. Tissues were harvested and plated for CFU at 2 weeks post-infection. Mean and SEM are shown. Each symbol represents one mouse, n ≥ 5. *p*-values for all experiments were determined as described in the methods. D) Comparison of colonization of the spleen and liver by the indicated strains. $P < 0.05$ (*)

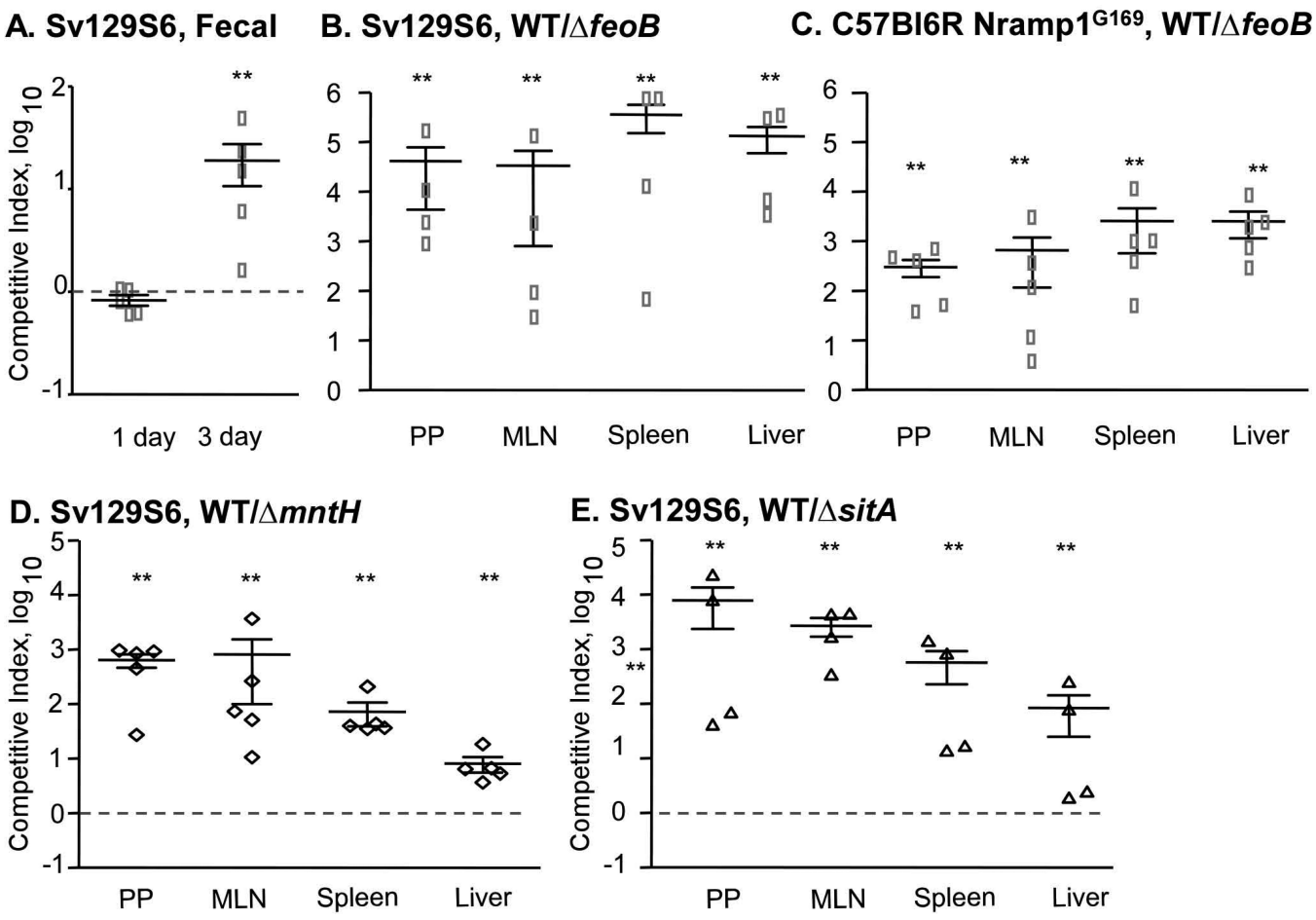

Figure 5. In mixed infections of mice, FeoB is required for *S*. Typhimurium replication

Mice were orogastrically inoculated with a 1:1 mixture of WT and Δ*feoB* strains (A-C), WT and Δ*mntH* strains (D), or WT and Δ*sitA* strains (E). Fecal pellets were harvested 1 and 3 days post-infection (A). Tissues from SV129S6 (B) or C57BL6 Nramp1^{G169} (C-E) mice were harvested and plated for CFU at 2 weeks post-infection. Mean and SEM of competitive index are shown; dashed lines indicate equivalent competition. *p*-values were determined as described in the methods. $p < 0.03$ (**) vs. the null hypothesis. Each symbol represents one mouse, n = 4-5.

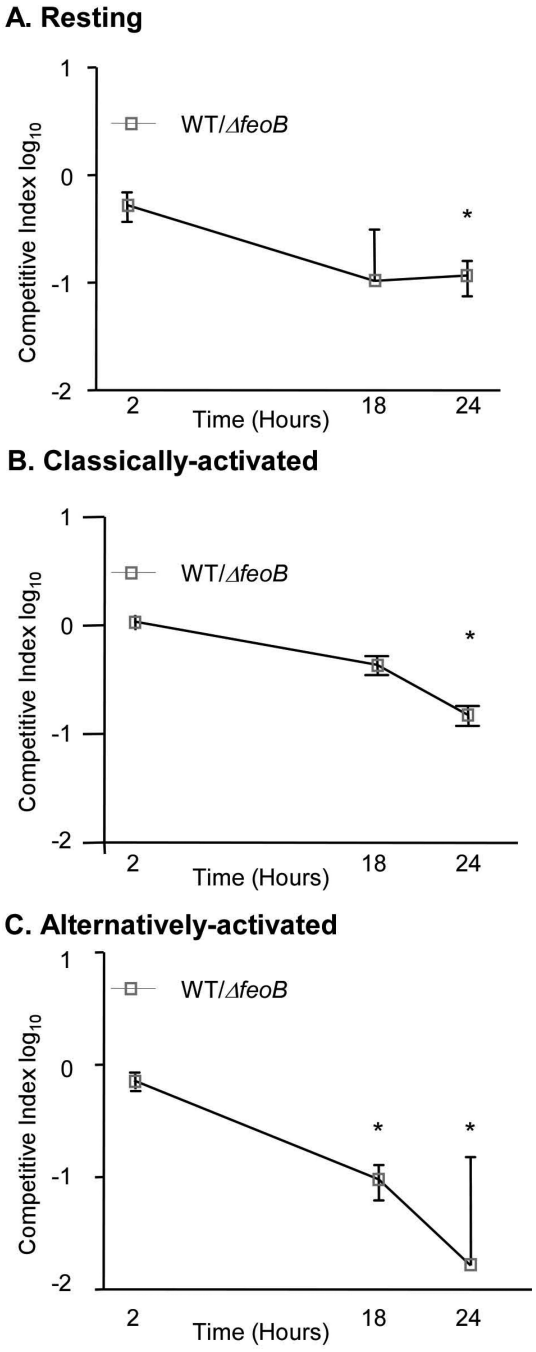

Figure 6. In mixed-infections of macrophages, FeoB limits *S*. Typhimurium replication

RAW264.7 Nramp1+ cells were resting (A) or treated with IFNγ and LPS (B) or IL-4 (C) and were inoculated with a 1:1 mixture of WT and Δ*feoB* strains. Mean and SD of representative experiments are shown. *p*-values were determined as described in the methods. $p < 0.05$ (*) vs. the null hypothesis, $n \geq 3$ experiments.

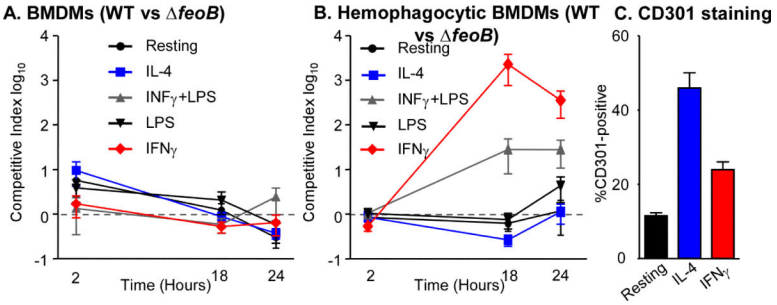

Figure 7. FeoB is required for replication upon mixed infection in BMDMs incubated with erythrocytes

A) BMDMs were resting or treated with IL-4, IFNγ, or IFNγ and LPS and then inoculated with a 1:1 mixture of WT and Δ*feoB* strains. Dashed line indicates equivalent competition. n ≥ 3 experiments. B) BMDMs were treated as in (A) and then incubated with erythrocytes. After one hour, cells were inoculated with a 1:1 mixture of WT and Δ*feoB* strains. Mean and SD of representative experiments are shown. Dashed line indicates equal competition. n ≥ 3 experiments. C) BMDMs were resting or treated with IL-4 or IFNγ for 18 hours. Cells were fixed and stained with anti-CD301 and analyzed by flow cytometry. Mean and SD of the percentage of BMDMs positive for CD301 from a representative experiment are shown. *p*-values were determined as described in the methods. $p < 0.005$ (*), n ≥ 3 experiments.

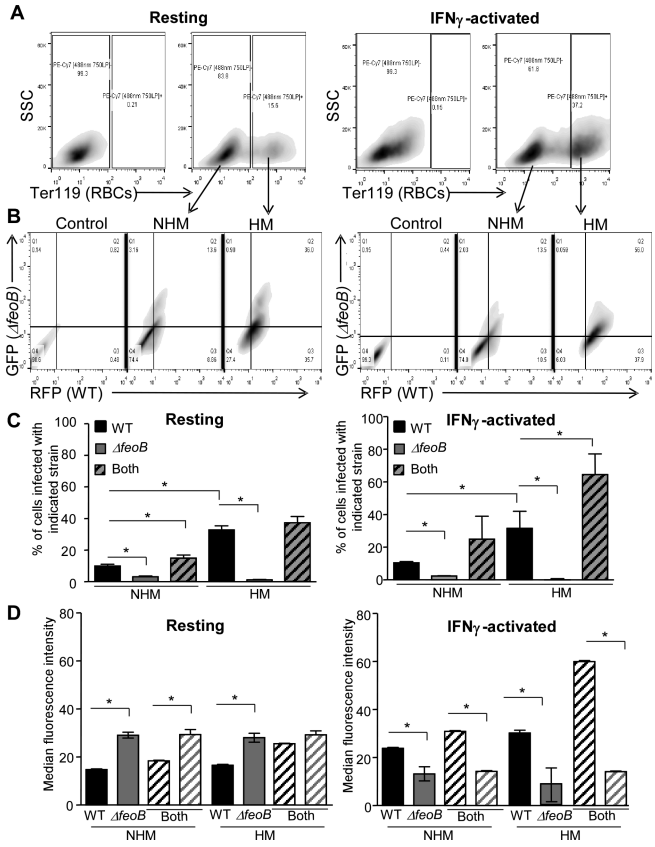

Figure 8. FeoB is required for replication upon mixed-infection in hemophagocytes

BMDMs were resting (A-D, left panels) or activated with IFNγ (A-D, right panels), incubated with erythrocytes for one hour, and then inoculated with a 1:1 mixture of WT-RFP and Δ*feoB*-GFP strains. At 18 hours post-infection, cells were fixed and stained with anti-Ter-119 and analyzed by flow cytometry. A) Gating scheme for non-hemophagocytic (NHM) and hemophagocytic (HM) populations. B) Gating scheme to identify RFP- and GFP-positive BMDMs. C) Percentage of BMDMs that were positive for WT (RFP, black), Δ*feoB* (GFP, gray) or both (striped). D) Median fluorescence intensity of WT and Δ*feoB* of singly and co-infected BMDMs from (C). Mean and SD of a representative experiment is shown. *p*-values were determined as described in the methods. $p < 0.05$ (*) vs. WT, $n \geq 3$.