RESEARCH ARTICLE

Salmonella enterica Infection Stimulates Macrophages to Hemophagocytose

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ABSTRACT **Hemophagocytes are cells of the monocyte lineage that have engulfed erythrocytes and leukocytes. Hemophagocytes frequently accumulate in patients with severe acute bacterial infections, such as those caused by *Salmonella enterica*, *Brucella abortus*, and *Mycobacterium tuberculosis*. The relationship between hemophagocytosis and infection is not well understood. In the murine liver, *S. enterica* serovar Typhimurium resides within hemophagocytic macrophages containing leukocytes. Here we show that *S.* Typhimurium also resides within hemophagocytes containing erythrocytes. In cell culture, *S.* Typhimurium benefits from residence within hemophagocytes by accessing iron, but why macrophages hemophagocytose is unknown. We show that treatment of macrophages with a cocktail of the proinflammatory cytokine interferon gamma (IFN-γ) and lipopolysaccharide (LPS) stimulates engulfment of nonsenescent erythrocytes. Exposure of resting or IFN-γ-treated macrophages to live, but not to heat-killed, *S.* Typhimurium cells also stimulates erythrocyte engulfment. Single-cell analyses show that *S.* Typhimurium-infected macrophages are more likely to erythrophagocytose and that infected macrophages engulf more erythrocytes than uninfected macrophages within the same culture well. In addition, macrophages containing erythrocytes harbor more bacteria. However, *S.* Typhimurium does not promote macrophage engulfment of polystyrene beads, suggesting a role for a ligand on the target cell. Finally, neither of the two *S.* Typhimurium type 3 secretion systems, T3SS1 or T3SS2, is fully required for hemophagocytosis. These results indicate that infection of macrophages with live *S. Typhimurium* cells stimulates hemophagocytosis.**

IMPORTANCE **Macrophages are white blood cells (leukocytes) that engulf and destroy pathogens. Hemophagocytes, a subset of macrophages, are characteristic of severe acute infection in patients with, for instance, typhoid fever, brucellosis, tuberculosis, and leishmaniasis. Each of these diseases has the potential to become chronic. Hemophagocytes (blood-eating cells) engulf and degrade red and white blood cells for unknown reasons. The bacterial pathogen *Salmonella* acquires the essential nutrient iron from murine hemophagocytes. We report that *Salmonella* stimulates macrophages to engulf blood cells, indicating that cells of this bacterium actively promote the formation of a specialized cellular niche in which they can acquire nutrients, evade killing by the host immune system, and potentially transition to chronic infection.**

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Serovars of the bacterium *Salmonella enterica* subsp. *enterica* cause self-limiting gastroenteritis or a systemic infection of macrophages, depending on the serotype-host combination. Gastroenteritis in humans or calves requires that *Salmonella enterica* serovar Typhimurium direct its own uptake by intestinal epithelial cells by secreting bacterial proteins into the host cell cytosol using a type 3 secretion system (T3SS1). Systemic infection of humans, typhoid fever, is caused by *S. enterica* serovar Typhi or Paratyphi, and requires colonization of macrophages and deployment of T3SS2, which regulates bacterial trafficking within host cells (1). *S.* Typhimurium infection of mice is a natural host-pathogen interaction that models human typhoid fever, including both the acute and chronic stages of disease (2).

One kind of macrophage that harbors *S.* Typhimurium during systemic infection is a hemophagocyte (HM) (3). HMs are derived from the process of hemophagocytosis ("blood eating"), histologically defined as the accumulation within tissues of monocytic cells containing engulfed erythrocytes and leukocytes. In healthy individuals, macrophages engulf and catabolize damaged and senescent cells. Hemophagocytosis is considered a distinct and pathological process in which hyperactivated macrophages or dendritic cells engulf multiple nonsenescent, intact blood cells. Observations of hemophagocytosis date from the late 1800s as a response to severe infection with bacteria, viruses, or parasites (4, 5). In typhoid patients, whether hemophagocytosis includes macrophages, dendritic cells, or both is unknown (6). In *S.* Typhimurium-infected mice, approximately 85% of splenic HMs express macrophage markers (7), as do hepatic HMs that harbor the bac-

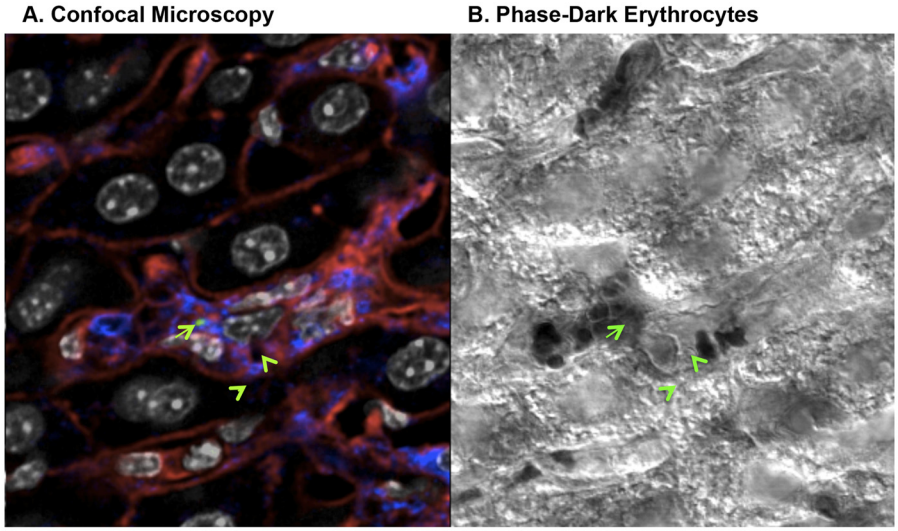

FIG 1 *S.* Typhimurium within a macrophage containing erythrocytes. (A) Confocal micrograph of a mouse liver 2 weeks post-oral gavage with 10^9 *S.* Typhimurium. *S.* Typhimurium (O-antigen, green arrows), macrophage (MOMA2, blue), actin (phalloidin, red), DNA (DAPI, white). The arrow points to a bacterium. Arrowheads point to bacteria in deeper planes of the macrophage (see Movie S1 in the supplemental material). (B) Erythrocytes are phase-dark, as visualized with differential interference contrast microscopy. A representative image is shown; $n = 4$ mice.

teria (3). In both humans and mice, HMs contain more erythrocytes than leukocytes (4, 8–11), possibly because erythrocytes are approximately 700 times more abundant. However, it has only thus far been reported that *S.* Typhimurium resides *in vivo* within HMs containing leukocytes (3).

The contributions of hemophagocytosis to disease progression are only now being elucidated. In a cell culture model, *S.* Typhimurium requires the ferrous iron transporter FeoB specifically in HMs containing engulfed erythrocytes (12). One possible explanation is that *S.* Typhimurium resides and replicates within HMs using nutrients derived from the breakdown of engulfed cells. Thus, HMs may be carriers for *S.* Typhimurium during the establishment and/or maintenance of chronic infection.

Proinflammatory cytokines have been speculated to drive hemophagocytosis because they are present during acute inflammation and because HM morphology is consistent with hyperactivation (13). One study demonstrated that sustained intraperitoneal delivery of the proinflammatory cytokine interferon gamma (IFN-γ) is sufficient to stimulate hemophagocytosis within 1 day in the murine spleen (14). However, hemophagocytosis also results from intraperitoneal or intravenous delivery of the innate immune stimulator CpG, the anti-inflammatory cytokine interleukin-4 (IL-4), or heat-killed *Brucella abortus* cells (15–18). Whether these agents act directly on macrophages to promote the engulfment of cells has not been established.

We demonstrate that *S.* Typhimurium resides within erythrocyte-containing HMs in the murine liver. In addition, we found unexpectedly that in cell culture, *S.* Typhimurium, but not *Escherichia coli*, stimulates macrophages to engulf erythrocytes. The bacteria must be viable and need to infect the macrophage, which will subsequently hemophagocytose. *S.* Typhimurium does not stimulate macrophages to engulf polystyrene beads, indicating specificity of engulfment. We observed more bacteria in HMs than in other macrophages, suggesting HMs support replication and/or survival of bacteria. The bacteria also do not require T3SS1 to drive hemophagocytosis, despite the role T3SS1 plays in promoting epithelial cells to engulf the bacteria (19). T3SS2 is also not needed, although the absence of both secretion systems modestly reduces hemophagocytosis. These results indicate that infection of macrophages with *S.* Typhimurium promotes hemophagocytosis and provides the bacterium with a survival niche *in vivo*.

RESULTS

***Salmonella* resides within macrophages containing erythrocytes in mice.** In humans and mice infected with *Salmonella*, HMs containing erythrocytes (erythrophagocytes) are more abundant than those containing leukocytes (8, 20, 21), but whether *S.* Typhimurium resides within the former has not been shown. We infected Sv129S6 mice orogastrically with 10^9 wild-type cells of *S.* Typhimurium strain SL1344 (22). Mice were euthanized after 2 or 3 weeks, and sections of the liver were harvested for microscopy and for tissue homogenization followed by plating of CFU. Tissue sections were fixed and stained with markers for macrophages (MOMA2 antibody), *S.* Typhimurium cells, DNA, and the actin cytoskeleton. Erythrocytes are difficult to observe by fluorescence microscopy because, despite perfusion of the animal with phosphate-buffered saline (PBS), the remaining extracellular erythrocytes cause significant background fluorescence (data not shown). However, under white light, erythrocytes are easily distinguishable because they appear phase dark due to the high density of heme (23). Confocal fluorescent and differential interference contrast microscopy revealed rod-shaped *S.* Typhimurium cells within macrophages containing multiple phase-dark erythrocytes (Fig. 1; see Movie S1 in the supplemental material). Macrophages have irregular shapes, and their cell boundaries are difficult to distinguish in three dimensions, even with a macrophage marker and actin staining. Therefore, we looked within the liver for bacterial signal, determined whether the bacterium appeared intact and rod-shaped, and then established whether the bacterium was within a macrophage. This process revealed that 157 of 171 (92%) intact bacterial rods were within macrophages containing phase-dark erythrocytes at 2 and 3 weeks postinfection ($n = 4$

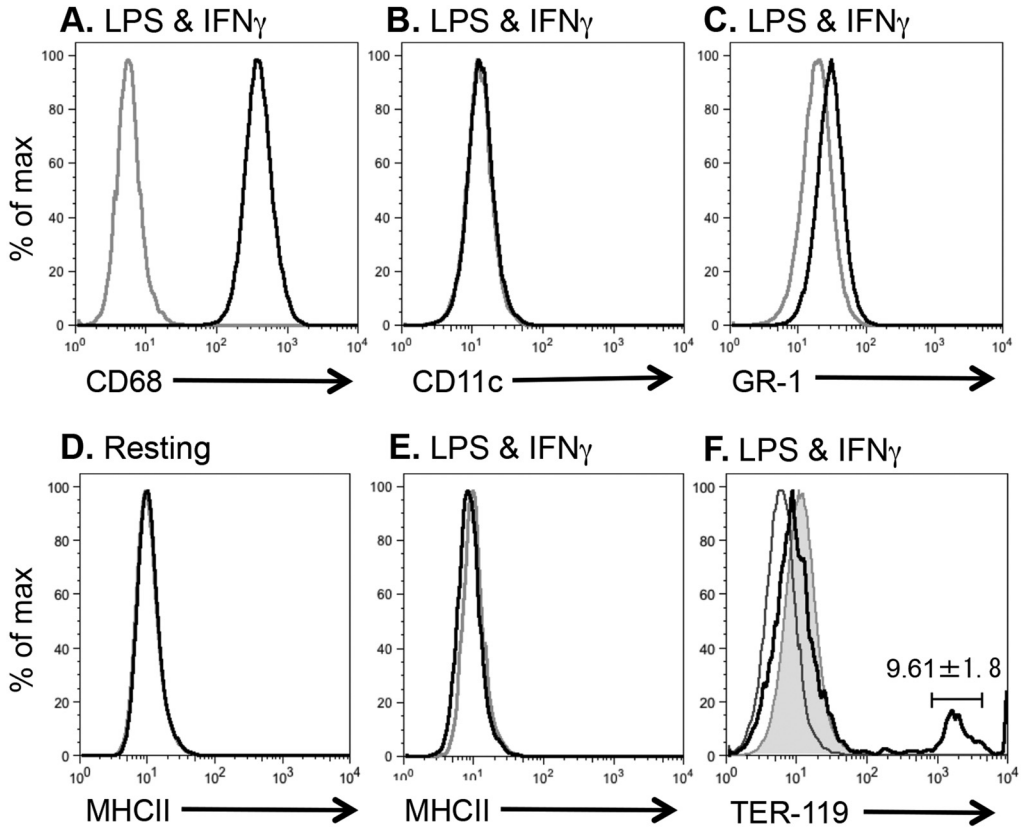

FIG 2 IFN-γ- and LPS-treated RAW267.4 Nramp1$^+$ macrophage-like cells engulf erythrocytes. Cells were treated with IFN-γ and LPS or medium alone (resting), as indicated, for 18 h and monitored by flow cytometry for markers characteristic of macrophages (CD68) (A), dendritic cells (CD11c) (B), and granulocytes (GR-1) (C). Resting (D) and IFN-γ- and LPS-treated (E) cells were examined for expression of MHC-II, a marker of activation. (F) IFN-γ- and LPS-treated cells were incubated with erythrocytes (10:1 erythrocyte/macrophage ratio) and monitored for erythrocyte engulfment (TER-119). (Macrophages unexposed to erythrocytes are shaded in gray.) Each experiment was repeated independently three or more times. Histograms from a representative experiment are shown. Gray lines, isotype controls; black lines, indicated markers.

mice). Thus, *S.* Typhimurium resides within erythrophagocytes in mice.

Macrophage-like cells erythrophagocytose upon treatment with IFN-γ and LPS. To examine erythrophagocytosis in cell culture, a previous report used a macrophage-like cell line, RAW264.7 (24). These cells are deficient for Nramp1 (Slc11a1), a cation transporter needed for normal iron regulation and containment of pathogens, including *S.* Typhimurium (25). We therefore examined erythrophagocytosis in a RAW264.7 line harboring an Nramp1^{G169} transgene that restores Nramp1 function (26, 27). Resting RAW264.7 Nramp1^{G169} cells expressed markers consistent with a macrophage phenotype: CD68$^+$, CD11c$^{low/-}$, and GR-1$^{int/-}$ (Fig. 2A to C). However, upon incubation with IFN-γ and lipopolysaccharide (LPS), RAW264.7 Nramp1^{G169} cells did not increase expression of major histocompatibility complex class II (MHC-II) (Fig. 2D and E), as expected (28). To determine the erythrophagocytic potential of RAW264.7 Nramp1^{G169}, we incubated cells with freshly isolated (nonsenescent) murine erythrocytes for 18 h. Prior to harvest, extracellular erythrocytes were lysed with hypotonic medium. We monitored macrophages for erythrophagocytosis by flow cytometry using an anti-TER-119 antibody, which recognizes cells of the erythrocyte lineage (Fig. 2F). Resting RAW264.7 Nramp1^{G169} cells exhibited no detectable erythrophagocytosis (data not shown), but approximately 10% of activated cells engulfed erythrocytes under these conditions (Fig. 2F). Thus, RAW264.7 Nramp1^{G169} cells are capable of engulfing erythrocytes.

Primary mouse macrophages treated with IFN-γ and LPS erythrophagocytose. We next examined primary mouse bone marrow-derived macrophages (BMDMs) from Sv129S6 mice, which are homozygous wild type for Nramp1^{G169}, as a potentially more authentic macrophage model system. IFN-γ- and LPS-treated BMDMs expressed macrophage markers and also had increased expression of MHC-II compared with resting BMDMs, as anticipated (Fig. 3A to E). BMDMs that were resting, stimulated with IFN-γ alone, or stimulated with IFN-γ and LPS were incubated with fresh erythrocytes and monitored for erythrophagocytosis by flow cytometry. IFN-γ and LPS pretreatment of BMDMs increased erythrophagocytosis 3-fold more than either medium or IFN-γ pretreatment alone, based on increased TER-119 signal (Fig. 3F and H). Erythrocyte engulfment also increased MHC-II expression (Fig. 3G). Compared with resting BMDMs, IFN-γ- and LPS-treated BMDMs engulfed 5-fold more erythrocytes over 18 h at erythrocyte/macrophage ratios ranging from 1:1 to 1:100 (Fig. 3I and J). Exposure to erythrocytes and/or erythrophagocytosis had no significant effect on the viability of resting or pretreated BMDMs (data not shown). These results indicate that

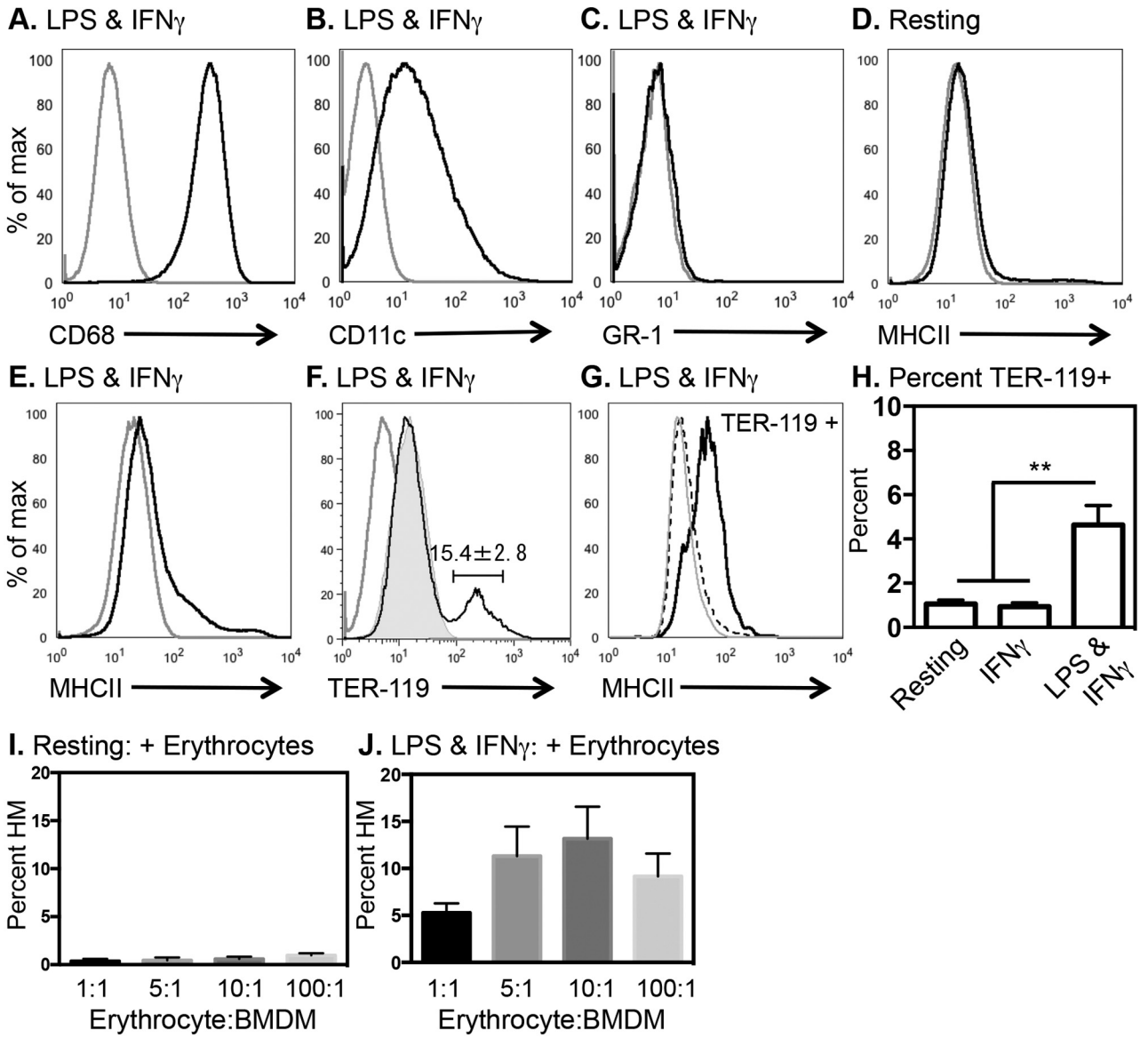

FIG 3 IFN-γ- and LPS-treated BMDMs engulf erythrocytes. BMDMs were treated with IFN-γ and LPS or medium alone (resting), as indicated, for 18 h and monitored by flow cytometry for markers characteristic of macrophages (CD68) (A), dendritic cells (CD11c) (B), and granulocytes (GR-1) (C). Resting (D) and IFN-γ- and LPS-treated (E) BMDMs were examined for expression of MHC-II, a marker of activation. (F) IFN-γ- and LPS-treated BMDMs were incubated with erythrocytes (10:1 erythrocyte/BMDM ratio) and monitored for erythrocyte engulfment (TER-119 [with BMDMs unexposed to erythrocytes shaded in gray]) and MHC-II expression (G) (dashed line, TER-119-BMDMs). (A to G) Each experiment was repeated independently three or more times. Histograms from representative experiments are shown. Gray lines, isotype controls; black lines, indicated markers. (H) Comparison of erythrocyte engulfment by differentially treated BMDMs ($n = 5$). The percentage of total resting ($n = 2$) (I) or IFN-γ- and LPS-treated (J) BMDMs ($n = 3$) with engulfed erythrocytes after an 18-h incubation is shown. Panels H to J represent aggregated data from multiple experiments with the mean and standard error of the mean (SEM). *P* values were determined as described in Materials and Methods. **, $P \leq 0.001$.

IFN-γ and LPS treatment together stimulates macrophages in cell culture to engulf erythrocytes.

Exposure to live *S.* Typhimurium cells stimulates erythrophagocytosis. The contribution of LPS to erythrophagocytosis (Fig. 3H) led us to examine whether whole bacteria could stimulate the process. BMDMs that were resting, treated with IFN-γ alone, or treated with IFN-γ and LPS were exposed to erythrocytes for 1 h prior to the addition of live *S.* Typhimurium cells (Fig. 4A to C). Within 18 h, the presence of bacteria stimulated macrophage uptake of erythrocytes 6-fold, 21-fold, and 3-fold, respectively, indicating that *S.* Typhimurium directly stimulates macrophages to hemophagocytose and has the largest effect on IFN-γ-treated cells.

LPS, *E. coli*, or heat-killed *S.* Typhimurium does not stimulate erythrophagocytosis. Macrophages respond to bacteria via recognition of microbial-associated molecular patterns (MAMPs), which stimulate proinflammatory pathways. To establish whether *S. Typhimurium* MAMPs are sufficient to promote erythrophagocytosis, we incubated BMDMs with erythrocytes and live or heat-killed *S.* Typhimurium cells, with commercial

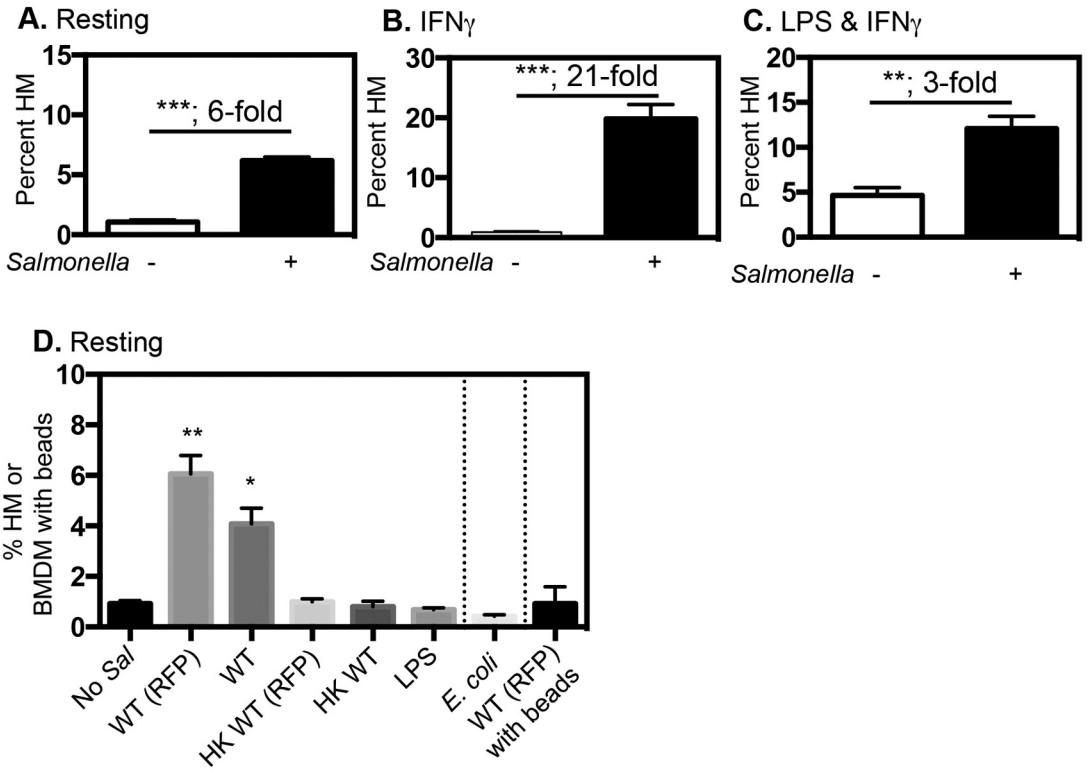

FIG 4 Incubation with live *S.* Typhimurium cells increases erythrophagocytosis. Resting ($n = 6$) (A), IFN-γ-treated ($n = 3$) (B), and IFN-γ-and LPS-treated (C) BMDMs ($n = 3$) were incubated with erythrocytes only (Ter-119$^+$ [white bar]) or erythrocytes and *Salmonella* (MOI,10; RFP [black bar]) for 18 h. (D) Resting BMDMs incubated with a 1:1 ratio of erythrocytes and with no bacteria (No Sal; $n = 3$), live *S.* Typhimurium (MOI, 10; $n = 5$), heat-killed (HK) *S.* Typhimurium (MOI, 10; $n = 3$ for HK WT and $n = 2$ for HK WT [RFP]), LPS ($n = 2$), or live *E. coli* cells (MOI 10; $n = 3$), as indicated. The rightmost bar represents resting BMDMs incubated with a 1:1 ratio of beads ($n = 3$). Aggregated data from multiple experiments are shown with the mean percentage of BMDMs that are TER-119$^+$ (or contain beads) and SEM. *P* values were determined as described in Materials and Methods. *, $P \leq 0.05$, **, $P \leq 0.001$, and ***, $P \leq 0.0001$, compared to incubation with no bacteria (No Sal).

S. Typhimurium-derived LPS, or with *E. coli*. Resting BMDMs were chosen for these experiments because macrophage stimulation with IFN-γ significantly increases bacterial cell death (29). Macrophages were exposed to an equivalent of 10 bacteria, live or dead, per macrophage or to an LPS equivalent of approximately 5,000 bacteria. Eighteen hours postinfection, only BMDMs incubated with live *S.* Typhimurium cells (with or without expression of a red fluorescent protein [RFP]) showed significant rates of erythrophagocytosis (Fig. 4D). Thus, macrophage exposure to high levels of *S.* Typhimurium-derived MAMPs or to *E. coli* is not sufficient to stimulate the uptake of erythrocytes.

***S.* Typhimurium does not stimulate macrophages to engulf inert beads.** Macrophage engulfment of particles can be mediated by signals from or ligands on the target particles. To establish whether *S.* Typhimurium stimulates BMDMs to engulf inert particles, which presumably lack such signals or ligands, we incubated BMDMs with 5.5-μm polystyrene beads or erythrocytes, which are approximately ~6 μm in diameter. Eighteen hours after infection with *S.* Typhimurium, a significant percentage of BMDMs engulfed the erythrocytes but not the beads (Fig. 4D, right). Therefore, macrophages, in response to *S.* Typhimurium, may recognize a ligand on the erythrocyte or possibly respond to soluble signal from the erythrocyte.

Infection with *S.* Typhimurium stimulates macrophages to erythrophagocytose. To establish whether *S.* Typhimurium needs to infect a macrophage to promote erythrocyte uptake by that macrophage, resting BMDMs were incubated with fresh erythrocytes and *S.* Typhimurium and monitored by flow cytometry for erythrocyte uptake and infection with bacteria. BMDMs that did not contain erythrocytes, as determined by a lack of TER-119 signal, are here referred to as nonhemophagocytes (NHMs). The percentages of infected and uninfected NHMs were similar (Fig. 5A, left). In contrast, 7-fold more HMs (TER-119$^+$) were infected than uninfected (Fig. 5A, right). Infected BMDMs contained more signal from erythrocytes than uninfected BMDMs (median fluorescent intensity of 10.0 versus 6.3, respectively) (Fig. 5B), suggesting that infection either increased the number of erythrocytes engulfed or delayed erythrocyte degradation. HMs contained twice as much bacterial signal as NHMs (75.3 versus 36.8) (Fig. 5C), supporting previous observations that HMs are permissive for *S.* Typhimurium (3, 30). All four classes of macrophages—HMs and NHMs, each with or without bacteria—were compared within the same culture well. Thus, infection with *S.* Typhimurium, and not simply exposure to bacteria or cytokines, stimulates erythrophagocytosis. Altogether, the data suggest that *S.* Typhimurium may directly manipulate macrophages to erythrophagocytose and provide a survival niche.

***S.* Typhimurium-induced erythrophagocytosis is T3SS1 and T3SS2 independent.** T3SS1 stimulates epithelial cells, which are normally nonphagocytic, to engulf *S.* Typhimurium cells, a key

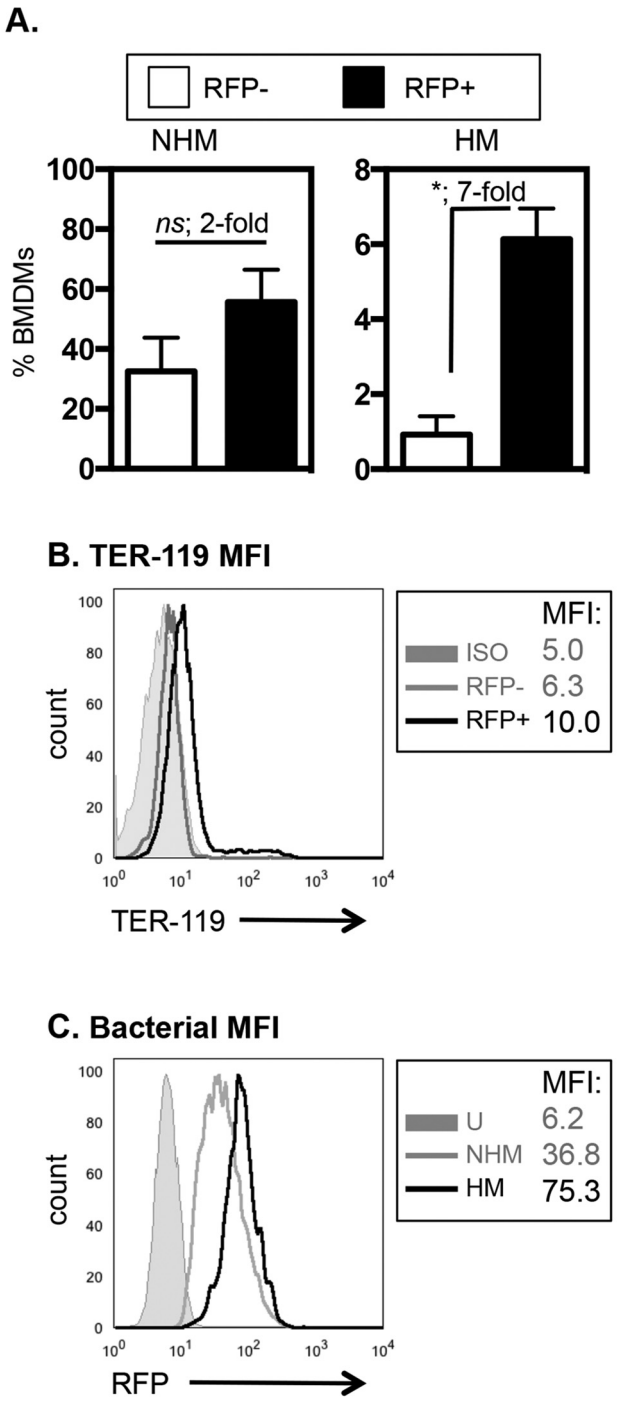

FIG 5 Infected BMDMs have increased erythrophagocytosis, and BMDMs containing erythrocytes support more *S.* Typhimurium cells. Resting BMDMs were incubated with *S.* Typhimurium cells expressing RFP and erythrocytes for 18 h. (A) Flow cytometry was used to define four distinct populations of BMDMs ($n = 6$). (Left) NHMs (Ter-119$^-$), RFP$^-$, or RFP$^+$. (Right) HMs (Ter-119$^+$), RFP$^-$, or RFP$^+$. Aggregated data from multiple experiments are shown with the mean percentages and SEM. (B) Representative histogram of the median fluorescence intensity (MFI) for TER-119 ($n = 3$). Gray shading, isotype (ISO) control; gray line, RFP$^-$ BMDMs; black line, RFP$^+$ BMDMs. (C) Representative histogram of the MFI for bacterial RFP ($n = 3$). Gray shading, uninfected (U) BMDMs; gray line, TER-119$^-$ (NHMs) BMDMs; black line, TER-119$^+$ (HMs) BMDMs. *P* values were determined as described in Materials and Methods. *, $P \leq 0.001$.

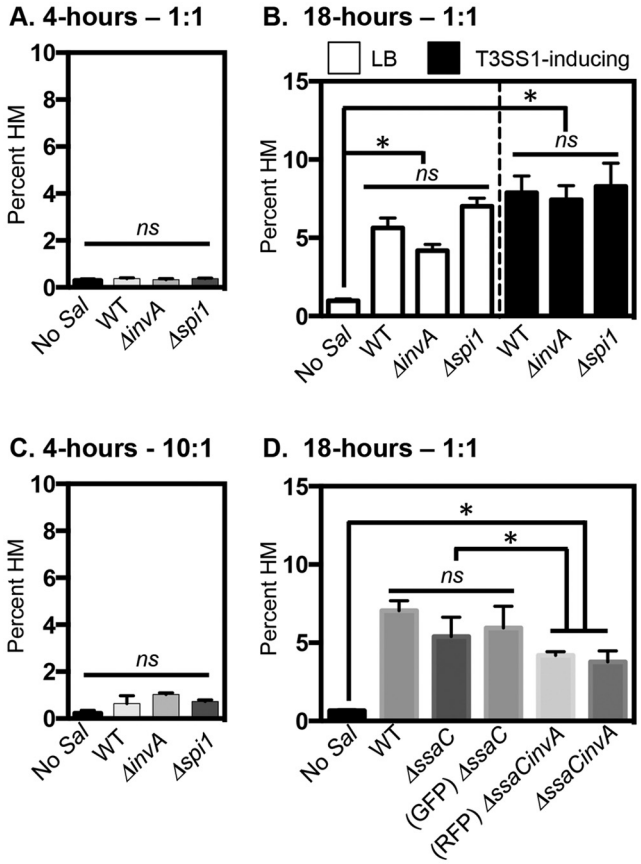

FIG 6 *Salmonella* stimulates erythrophagocytosis independently of T3SS1 or T3SS2. Primary macrophages were incubated with erythrocytes only (1:1) or erythrocytes (1:1) and bacteria (MOI, 10) for 4-h T3SS1 mutants ($n = 3$) (A), 18-h T3SS1 mutants ($n = 4$ for LB and 3 for T3SS1-inducing conditions) (B), 10:1 erythrocytes for 4-h T3SS1 mutants ($n = 2$) (C), or 18-h T3SS1 and T3SS2 mutants ($n = 3$) (D). Aggregated data from multiple experiments are shown with the mean percentages of BMDMs that are TER-119$^+$ and SEM. *P* values were determined as described in Materials and Methods. *, $P \leq 0.01$.

step of enteritis (2, 19). We therefore hypothesized that *S.* Typhimurium may use T3SS1 to promote erythrophagocytosis. However, macrophages did not begin erythrophagocytosis until at least 4 h after infection, whether the erythrocyte/macrophage ratio was 1:1 or 10:1 (Fig. 6A and C). While T3SS1 is best known for secreting proteins into host cells before and shortly after infection, it remained possible that T3SS1 stimulates erythrophagocytosis because some T3SS1-secreted proteins, such as SipA, function after infection to modulate phagosomal properties (31, 32). To test for a possible role in erythrophagocytosis of T3SS1 and also T3SS2 (a later-acting secretion system that remodels the phagosome), mutant strains were examined. The *invA*::*cm* and *ssaC spiA*::*kan* strains lack genes encoding core machinery components of T3SS1 and T3SS2, respectively (33). The *spi1*::*kan* strain lacks a large portion of T3SS1 (34), and strain P7G2 has an inactivating insertion in *ssaC* and *spiA* (35). By 18 h postinfection, wild-type and T3SS single mutant strains stimulated macrophages to erythrophagocytose (Fig. 6B and D). Curiously, T3SS1 T3SS2 double mutant strains were modestly compromised for induction of erythrophagocytosis. Therefore, a machinery component(s) (36) or an effector(s) that can be secreted from either apparatus (37) may contribute to triggering hemophagocytosis in BMDMs.

DISCUSSION

Our results show that *S.* Typhimurium resides in the liver of Sv129S6 mice within hemophagocytes (HMs) containing erythrocytes. This finding complements previous observations of *S.* Typhimurium within HMs containing leukocytes (3). Sv129S6 mice develop an acute infection that transitions to chronic infection (38). Infected and uninfected multinucleate HMs are apparent in the liver by confocal microscopy with 4′,6-diamidino-2-phenylindole (DAPI) and by phase-contrast microscopy at 1, 2, and 3 weeks postinfection. The nuclei within the macrophages appear to be intact and are surrounded by actin, consistent with engulfment and hemophagocytosis (3). Actin was also useful for discerning the edges of cells, as macrophages are large and irregularly shaped. Fluorescence and phase-contrast microscopy show a combination of leukocytes (3) and erythrocytes within hepatic HMs. In the spleen, macrophages containing T cells and erythrocytes and also large amounts of intact DNA (>6 N) are detectable by flow cytometry (7). Altogether, these data suggest that *S.* Typhimurium-infected Sv129S6 mice accumulate HMs and that the bacteria reside within HMs.

It is interesting that HMs have not been reported in widely studied *S.* Typhimurium acute infection models, including the C57BL/6 and BALB/c mouse strains (39, 40). These mice develop acute infection that is fatal within a week, in contrast to the chronic infection of the Sv129S6 mice used in this study. In a brief examination of *S.* Typhimurium-infected BALB/c mouse livers, we did not observe significant numbers of multinucleated macrophages, although at that time, we did not try to visualize erythrocytes by phase-contrast microscopy (unpublished data). It is conceivable that HMs do not accumulate in mice with severe fatal acute infection. For instance, splenic HMs do not accumulate in Sv129S6 mice within a week of oral inoculation with *Yersinia pseudotuberculosis*, which is fatal by 9 to 10 days (7). Whether HMs accumulate in mice upon infection with other bacterial pathogens remains to be determined.

In resting macrophages, infection with live *S.* Typhimurium cells increased the percentage of HMs. Infected HMs contained more erythrocytes than uninfected HMs, and HMs had more bacteria within them than NHMs. Exposure to high levels of LPS or to *E. coli* or heat-killed *S.* Typhimurium was not sufficient to stimulate hemophagocytosis. In addition, *S.* Typhimurium promotes erythrophagocytosis independently of T3SS1, which is curious in light of the requirement for T3SS1 in stimulating epithelial cell engulfment of the bacteria (41). T3SS2 is also not required, but elimination of both secretion systems modestly impaired erythrophagocytosis. This observation suggests that an effector(s) that can be secreted by either system or machinery components detected by the host could play a role. We further speculate that additional innate immune system stimuli, such as flagellin and LPS, contribute to the promotion of hemophagocytosis.

In our cell culture model, the percentage of HMs after infection with *S.* Typhimurium increased most dramatically in IFN-γ-pretreated macrophages, compared to macrophages treated with a combination of IFN-γ and LPS. Differential pretreatment did not correlate with different infection rates (data not shown). Accordingly, pretreatment with LPS reduces the ability of macrophages to respond to a later exposure to LPS, a phenomenon referred to as "tolerance" (42). Thus, although IFN-γ- and LPS-treated macrophages have the highest uninfected percentage of HMs, they have the smallest response to live *S.* Typhimurium cells because infection after priming with LPS does not stimulate appropriate signaling pathways. It does not appear that macrophage cytokines acting in a paracrine manner play a significant role in promoting hemophagocytosis, as uninfected macrophages in the same culture well with infected macrophages did not engulf more erythrocytes.

It has been suggested that *in vivo*, proinflammatory cytokines stimulate macrophages to hemophagocytose. Support for this idea includes observations that high levels of serum proinflammatory cytokines correlate with hemophagocytosis in humans and mice and that HM morphology suggests hyperactivation (13). Moreover, in mice, sustained delivery of IFN-γ to the peritoneal cavity results in HM accumulation in the spleen within a day (14). Splenic HMs also accumulate upon peritoneal delivery of the anti-inflammatory cytokine IL-4, the bacterial product CpG (unmethylated), or heat-killed *Brucella abortus* (15–17). In the blood, cells of the monocyte lineage ($CD11c^+$) that engulf erythrocytes accumulate in response to intravenous delivery of CpG or poly(I-C) (18). These $CD11c^+$ cells engulfed circulating erythrocytes via recognition of phosphatidylserine (18), consistent with our observation that live *S.* Typhimurium cells stimulate bone marrow-derived macrophages in culture to engulf erythrocytes but not polystyrene beads. However, it is unclear whether any of the cytokines or innate immune system stimulators delivered into mice act directly or indirectly on macrophages to promote hemophagocytosis. Our data suggest that in primary mouse macrophages, IFN-γ treatment without subsequent infection is not sufficient to stimulate erythrophagocytosis. In contrast, treatment with both IFN-γ and LPS stimulates macrophages to engulf either erythrocytes or leukocytes (3). In summary, (i) treatment of macrophages with IFN-γ and LPS (in the absence of infection) and (ii) infection with *S.* Typhimurium are both sufficient to directly stimulate hemophagocytosis. These observations suggest macrophages require multiple signals to hemophagocytose and provide a tractable model system for understanding mechanism.

A remaining question is whether erythrophagocytosis, by removing erythrocytes from circulation, contributes to the regenerative, microcytic anemia that develops in severely infected mice (8, 14, 43). This is of interest in part because anemia is also a clinical sign of human typhoid fever (44). Confocal microscopy in mice suggests that many or most hepatic macrophages are HMs, but flow cytometry identifies only 3 to 5% of splenic macrophages as HMs (7). This discrepancy may reflect differences in tissue biology, methodology, or both. During the cell separation steps essential for flow cytometry, some spleen cells are destroyed—particularly larger cells such as HMs. However, it is difficult to quantitatively analyze splenic red pulp by microscopy because the cells have very irregular shapes and are highly interdigitated, such that tracing the edges of large cells, such as macrophages, is extremely time-consuming. Therefore, we generally use flow cytometry to analyze the spleen. The liver is ideal for confocal microscopy because hepatocytes are of regular shape (cuboidal). However, the connective tissue within the liver makes it difficult to separate hepatocytes for flow cytometry. Regardless of how prevalent HMs are in the liver versus the spleen, circumstances other than erythrophagocytosis likely contribute to anemia in *S.* Typhimurium-infected mice. Infected mice have decreased platelets, reduced serum fibrinogen, fragmented erythrocytes, and thrombosis (8). As infection progresses, erythrocytes may become damaged upon passage through blood clots and may be captured

by macrophages. In addition, high levels of serum IFN-γ and tumor necrosis factor alpha (TNF-α) for 6 weeks after infection (unpublished data) likely contribute to erythrocyte oxidation and engulfment by macrophages (45) and also to decreased erythroid production (46). Thus, hemophagocytosis is likely to be only one of multiple factors contributing to anemia in murine typhoid fever.

Macrophage ingestion of blood cells in response to IFN-γ and LPS or to *S.* Typhimurium infection may have allowed the bacteria to evolve to survive within HMs. There are at least two advantages HMs may offer *S.* Typhimurium compared to other macrophages. First, the bacteria acquire iron, an essential nutrient from HMs (12). Second, HMs may provide a less toxic environment for the bacteria than other macrophages. Degradation of heme to release iron also releases carbon monoxide and biliverdin, which are anti-inflammatory. In neutrophils, heme degradation interferes with the oxidative burst, thereby allowing *S.* Typhimurium to survive within these otherwise lethal cells (47). It is therefore possible that the anti-inflammatory properties of carbon monoxide and biliverdin contribute to the alternative, noninflammatory (M2) activation state of HMs (7, 48). M2 macrophages, and possibly HMs, can also provide *S.* Typhimurium with glucose (49). In *S.* Typhimurium-infected mice, HMs are M2 when most other splenic macrophages are classically (M1) activated and proinflammatory (7). The anti-inflammatory properties of M2 macrophages may benefit the host during acute infection: in a baboon *E. coli* sepsis model, M2 macrophages correlate with survival, whereas M1 macrophages dominated in nonsurvivors (50). In the context of systemic *Salmonella* infection, M2 macrophages, including HMs, may reduce inflammation but also provide the bacteria with a refuge from the immune system and a source of nutrients (3, 12, 49), allowing the pathogen to ultimately establish chronic infection. In support of this idea, engulfment of erythrocytes by circulating cells of the monocyte lineage led to production of IL-10, which reduced tissue damage and mouse mortality (18). A major outstanding question is whether other bacteria that cause both hemophagocyte accumulation and chronic infection, such as *Mycobacterium tuberculosis* and *Brucella abortus*, also stimulate macrophages to hemophagocytose and survive within HMs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type strain was *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 (22). ESH829 was the wild-type strain SL1344 transformed with pRFPTag (pRFPTag is pTag expressing the monomeric red fluorescent protein [RFP] and was a gift from the A. Palmer lab). The relevant characteristics of the mutant strains are as follows: KDE565, SL1344 *invA*::*cm* (51); CSD163/RM69, SL1344 *spi1*::*kan* in which *Salmonella* pathogenicity island 1 (SPI1) from *invH* to *avrA* was replaced with a kanamycin resistance marker (34, 52); P7G2, *ssaC/spiA*::mTn*5*-GFP-*kan* (35); SCS480, *ssaC/spiA*::*kan* (30); and SCS663, *invA*::*cm ssaC*::*kan* (30). Strain SCS663 contains the pRFPTag. *Escherichia coli* DH5α was obtained from Life Technologies, Grand Island, NY. Strains were grown overnight at 37°C with aeration prior to infections. For SPI1-inducing conditions, bacteria were grown overnight at 37°C without agitation (53). Antibiotics were used at the following concentrations: streptomycin, 30 μg/ml; kanamycin, 30 μg/ml; chloramphenicol, 20 μg/ml; and ampicillin, 300 μg/ml.

Mouse infections and tissue collection. Research protocols were approved by the University of Colorado Institutional Committees for Biosafety and for Animal Care and Use. For mouse infection studies, 7-week-old male 129SvEvTac (Nramp1$^{G169/G169}$) mice (Taconic Laboratories, Hudson, NY) bred in-house were fasted for 2 to 4 h prior to orogastric inoculation with 1×10^9 wild-type SL1344 bacteria, as verified by plating for CFU on selective LB agar, in 100 μl of phosphate-buffered saline (PBS: 10 mM phosphate buffer, 150 mM NaCl [pH 7.4]). Two or 3 weeks after inoculation, mice were euthanized and perfused with 20 ml ice-cold PBS. Portions of the spleen and liver were weighed and homogenized in 1 ml PBS with a TissueMiser (Fisher Scientific) and diluted in PBS for plating on selective LB agar plates. Liver portions were placed in 4% paraformaldehyde in 1× PBS (pH 7.4) and postfixed overnight. The following day, 50-μm consecutive liver sections were cut using a vibratome (Leica Microsystems, Bannockburn, IL).

Immunohistochemistry and image collection. Vibratome sections were washed in PBS 2 times for 15 min and then placed in blocking solution (1% bovine serum albumin, 1% normal goat serum, 1% glycine, 0.4% Triton X-100 in PBS) for 48 h at 23°C. Slices were placed in primary antibody at 4°C for 48 h: rat anti-MOMA2 (MCA519; Serotech) at 1:500 and rabbit anti-O-antigen (240984; BD) at 1:200. The following day, slices were washed in PBS with 0.4% Triton X-100 5 times for 30 min and placed in secondary antibody at 23°C for 24 h: goat anti-rabbit Alexa Fluor 488 (A11008; Molecular Probes, Carlsbad, CA) at 1:1,000 and goat anti-rat Alexa Fluor 555 (A21434; Molecular Probes) at 1:1,000. Slices were also stained with Alexa Fluor 647-phalloidin (A22287; Molecular Probes) at 1:200. The following day, the slices were washed in PBS with 0.4% Triton X-100 5 times for 30 min. Slices were stained with 4',6-diamidino-2-phenylindole dilactate (DAPI) (D3571; Molecular Probes) at 1:1,000 for 20 min and then washed 3 times for 10 min in PBS. Slices were mounted on slides with a no. 1.5 cover glass in Fluoromount-G (Southern Biotech, Birmingham, AL). Confocal z-stacks were acquired using a Zeiss LSM 510 confocal system with a 40× oil NA 1.3 objective (Carl Zeiss, Germany) (pixel size, 0.09 μm/pixel).

BMDM generation and RAW264.7 Nramp1^{G169} culture. Primary macrophages were derived as previously described (3). Briefly, marrow was flushed from the femurs and tibias of 2- to 4-month-old 129SvEvTac mice (Taconic Laboratories, Hudson, NY). Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with fetal bovine serum (10%), L-glutamine (2 mM), sodium pyruvate (1 mM), β-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 500 × *g* for 15 min. Monocytes at the interface were harvested and incubated for 6 days at 37°C in 5% CO_2 in supplemented DMEM that also contained 30% macrophage colony-stimulating factor (M-CSF) (conditioned medium from 3T3-MCSF cells) to promote differentiation of adherent monocytes into macrophages (54). BMDM and RAW264.7 Nramp1^{G169} cells were activated with 20 ng/ml *S. enterica* serovar Typhimurium LPS from Sigma-Aldrich (where the dose is equivalent to a multiplicity of infection [MOI] of ~5,000 [20 pM] based on an *E. coli* dry weight of 2.8e−13 g/cell [55], 4.09 nmol LPS/mg dry weight of *S. Typhimurium* [56], and the formula weight of *S. Typhimurium* LPS [Sigma-Aldrich]) and 20 U/ml IFN-γ (PeproTech, Rocky Hill, NJ) or with only 20 U/ml IFN-γ for 18 h.

Macrophage uptake of erythrocytes or beads, with or without bacterial infection. BMDMs were isolated as described above. Resting or activated (20 ng/ml LPS and 20 U/ml IFN-γ or only 20 U/ml IFN-γ for 18 h) BMDM or RAW264.7 Nramp1^{G169} cells were seeded at 4×10^5 cells per well in a 6-well plate (80 to 100% confluence) and incubated with medium alone, various concentrations of freshly isolated murine erythrocytes (obtained by cardiac puncture, harvested by centrifugation for 10 min, and added to macrophages within 30 min of acquisition) or inert microspheres (internally dyed with Starfire Red [size, 5.5 μm]; Bangs Laboratories, Inc., Fishers, IN) for 1 h. Where indicated, live bacteria, heat-killed bacteria (at an MOI of 10 with regard to macrophages), or 20 ng/ml LPS was added. After 30 min, cells were washed and incubated for 1.5 h at 37° C in fresh medium supplemented with gentamicin (100 μg/ml) to kill extracellular bacteria. Medium was then exchanged for

medium supplemented with gentamicin (10 μg/ml) to prevent extracellular bacterial growth. Erythrocytes or inert microspheres were resupplied at the same concentration after each change of medium. At 4, 18, or 24 h, wells were washed twice with cold PBS, and erythrocytes were lysed with ammonium chloride-potassium bicarbonate (ACK) lysis buffer (57) and harvested by gentle scraping for flow cytometry. BMDM cell death was monitored in resting and activated (LPS and IFN-γ) macrophages 18 h after the addition of various concentrations of freshly isolated murine red blood cells (RBC) using the LIVE/DEAD fixable near-infrared (IR) dead cell stain following the manufacturer's protocol (Life Technologies, Grand Island, NY) and analyzed by flow cytometry.

Flow cytometry. Resting or activated (20 ng/ml LPS and 20 U/ml IFN-γ or only 20 U/ml IFN-γ for 18 h) BMDM or RAW264.7 Nramp1^{G169} cells were harvested by gentle scraping. Cells from each condition were equally distributed into 96-well plates and resuspended in fluorescence-activated cell sorter (FACS) staining buffer (PBS plus 1% fetal bovine serum [FBS], 0.02% azide) containing anti-mouse CD16/32 (eBioscience, San Diego, CA) to block Fc receptors. Cells were then fixed on ice in 1% paraformaldehyde–1% sucrose, permeabilized in staining buffer with 0.1% saponin for 10 min, and then incubated in permeabilization buffer containing 1:50 anti-mouse CD68-RPE (FA11; AbD Serotec, Raleigh, NC) (isotype, rat IgG2a-RPE; AbD Serotec), 1:200 anti-mouse CD11c-allophycocyanin (APC) (isotype, Armenian hamster IgG-APC; eBioscience), 1:200 anti-mouse GR-1-phycoerythrin (PE)-Cy7 (RB6-8C5) (isotype, rat IgG2b K-PE-Cy7; eBioscience), or 1:200 anti-mouse MHC-II-fluorescein isothiocyanate (FITC) (isotype, rat IgG2b K-FITC; 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., Ashland, OR). Cells incubated with freshly isolated murine red blood cells (RBC) were analyzed after 18 h also using 1:200 anti-mouse TER-119-APC (isotype, rat IgG2b K-APC; eBioscience).

Statistics. *P* values were calculated with GraphPad Prism 6 (GraphPad Software, Inc.) and considered significant at $P < 0.05$. For nonparametric data, Wilcoxon's signed-rank test or Mann-Whitney test was used. Otherwise, Student's *t* test or analysis of variance (ANOVA) was used.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02211-14/-/DCSupplemental.

Movie S1, AVI file, 11.2 MB.

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