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Hemophagocytic Macrophages in Murine Typhoid Fever Have an Anti-Inflammatory Phenotype

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Histiocytes are white blood cells of the monocytic lineage and include macrophages and dendritic cells. In patients with a variety of infectious and noninfectious inflammatory disorders, histiocytes can engulf nonapoptotic leukocytes and nonsenescent erythrocytes and thus become hemophagocytes. We report here the identification and characterization of splenic hemophagocytes in a natural model of murine typhoid fever. The development of a flow-cytometric method allowed us to identify hemophagocytes based on their greater than 6N (termed 6N+) DNA content. Characterization of the 6N+ population from infected mice showed that these cells consist primarily of macrophages rather than dendritic cells and contain T lymphocytes, consistent with hemophagocytosis. Most 6N+ macrophages from *Salmonella enterica* serovar Typhimurium-infected mice contain intact DNA, consistent with hemophagocytosis. In contrast, most 6N+ macrophages from control mice or mice infected with a different bacterial pathogen, *Yersinia pseudotuberculosis*, contain damaged DNA. Finally, 6N+ splenic macrophages from *S.* Typhimurium-infected mice express markers consistent with an anti-inflammatory M2 activation state rather than a classical M1 activation state. We conclude that macrophages are the predominant splenic hemophagocyte in this disease model but not in *Y. pseudotuberculosis*-infected mice. The anti-inflammatory phenotype of hemophagocytic macrophages suggests that these cells contribute to the shift from acute to chronic infection.

Salmonella enterica is a Gram-negative bacterial species that causes enteric or systemic infection. In humans, *S. enterica* serotypes Typhi and Paratyphi A to C cause typhoid fever, a systemic infection of the reticuloendothelial system. The bacteria are acquired orally through contaminated food and water. Acute typhoid fever resolves into a chronic infection in approximately 4% of individuals. Chronic infection is a public health issue, because bacterial shedding in the feces ensures the maintenance of the pathogen in a population. As with other microbes, clinical multidrug-resistant *S. enterica* strains have emerged in recent years and are cause for concern (25).

Humans with typhoid fever accumulate hemophagocytic histiocytes, macrophages, or dendritic cells that engulf intact leukocytes and erythrocytes. Hemophagocytosis is associated not only with typhoid fever but also with other infections, such as leishmaniasis, tuberculosis, histoplasmosis, and influenza (14). Hemophagocytes also accumulate in patients with inflammatory disorders, such as macrophage activation syndrome (MAS). In typhoid fever patients, hemophagocytes were described in the late 1800s upon autopsy: "numerous large phagocytic cells containing usually many red blood (cell) globules or undigested portions of them, less frequently lymphoid cells" (19). At that time, macrophages and dendritic cells were not recognized as distinct cell types. More recent studies have confirmed the presence of hemophagocytes within the bone marrow of typhoid fever patients, but the identity of these cells has not been established with cell-type-specific markers (17, 34). Whether the hemophagocytes of typhoid fever are macrophages, dendritic cells, or both is of potential clinical significance, because histiocytoses are currently classified and treated based on the origin of the histiocytes (10).

Mice are a natural host for *S. enterica* serotype Typhimurium, which causes a systemic infection that models human typhoid fever. *S.* Typhimurium resides within macrophages, a professional phagocyte that can have pro- or anti-inflammatory properties (30). In the liver of infected mice, *S.* Typhimurium was observed within hemophagocytic macrophages that were identified by confocal microscopy using multiple cell type-specific antibodies (24). Hemophagocytic histiocytes, either macrophages or dendritic cells, were also demonstrated in bone marrow cytology and spleens of infected Sv129S6 mice (6). Here, we develop a quantitative method to identify hemophagocytes within a mouse model of typhoid fever, determine that the hemophagocytes in this model consist primarily of macrophages, and show that they express markers consistent with an anti-inflammatory phenotype.

MATERIALS AND METHODS

Bacteria. *Salmonella enterica* serovar Typhimurium (strain SL1344; *hisG*, *xyl*, *rpsL*) (35) or *Yersinia pseudotuberculosis* strain IP2666 (5) were grown overnight at 37 or 26°C, respectively, in Luria-Bertani broth with aeration. Upon plating, streptomycin (30 μg/ml) was used to select for *Salmonella*, and irgasan (2 μg/ml) was used for *Y. pseudotuberculosis* (2).

Mice and infections. Research protocols were approved by the University of Colorado Institutional Biosafety and Animal Care and Use Committees. Seven-week-old Sv129S6/SvEvTac mice (Taconic Farms, Hudson, NY) bred in house were fasted for 2 to 3 h prior to infection and were housed separately from mock-infected control mice. Mice were infected orogastrically with 100 μl of phosphate-buffered saline (PBS) containing 1×10^9 to 4×10^9 CFU of *S.* Typhimurium or 0.6×10^9 to 2×10^9 CFU of *Y. pseudotuberculosis* as determined by plating. Control mice received PBS.

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Flow cytometry. Murine spleens were mechanically dispersed and passed through a 70-μm-pore-size cell strainer into 10 ml of Dulbecco's modified Eagle medium (DMEM). An aliquot of each suspension was lysed for 20 min with NP-40 (0.2%) and plated for bacterial CFU. No CFU were found in control animals. Remaining cells were washed and passed through a 40-μm-pore-size cell strainer. An aliquot of cells was treated with hypotonic lysis buffer to eliminate erythrocytes and counted on a hemocytometer. Remaining cells were distributed into 96-well plates at 1×10^6 cells per well and resuspended in 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 stained extracellularly with anti-mouse CD68-RPE (FA11; AbD Serotec, Raleigh, NC) (18, 28). Cells were additionally stained with anti-mouse CD11c-allophycocyanin (APC) and anti-mouse Gr-1-phycoerythrin (PE)-Cy7 (RB6-8C5; eBioscience) and other antibodies listed below. The CD68 isotype control was rat IgG2a-RPE (AbD Serotec). A subset of murine kidney dendritic cells express surface CD68 (33). Therefore, the integrin CD11c (3) distinguished macrophages from dendritic cells. Cells were fixed on ice in 1% paraformaldehyde–1% sucrose, permeabilized in staining buffer with 0.1% saponin, and then incubated in staining buffer containing 10 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), 0.1% saponin. Fluorescently labeled cells were quantified with a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA) and analyzed with appropriate isotype controls and compensation using FlowJo software (Tree Star, Inc., Ashland, OR). Macrophages were gated on Gr-1^{int} to distinguish them from neutrophils, which are Gr-1^{high} (15). Hemophagocytic $CD68^+$ cells (macrophages) were consistently within the Gr-1^{int} gate. To establish whether T lymphocytes were within cells, dissociated spleen cells were stained with anti-mouse T cell receptor (TCR)β-APC (Biolegend, San Diego, CA) before or after permeabilization. The isotype control antibody was not useful because the background staining associated with the anti-TCRβ antibody was higher than that associated with the isotype control and other anti-TCRβ antibodies conjugated to different fluorophores (data not shown). To identify cells containing fragmented DNA, the APO-BRDU kit was used per the manufacturer's instructions (Phoenix Flow Systems, San Diego, CA). Two infected mice, one at 2 and one at 3 weeks postinfection, were eliminated from the time course analyses because upon sacrifice, (i) tissue colonization was below the limit of detection and (ii) more than 85% of 6N+ macrophages were bromodeoxyuridine positive ($BrdU^+$). Antibodies to additional cell surface markers were rat anti-mouse CD206-fluorescein isothiocyanate (FITC) (Biolegend), Armenian hamster anti-mouse CD80-FITC, rat anti-mouse CD150-APC, rat anti-mouse CD200-Alexa Fluor 647, rat anti-mouse CD200R-APC (eBioscience), rat anti-mouse CD204-Alexa Fluor 488, and rat anti-mouse CD36-Alexa Fluor488 (AbD Serotec). Isotype controls included rat IgG2a-FITC (Biolegend), Armenian hamster IgG-FITC, rat IgG2b-APC, rat IgG2a-Alexa Fluor 647, rat IgG2a-APC (eBioscience), rat IgG2b-Alexa Fluor 488, and rat IgG2a-Alexa Fluor 488 (AbD Serotec). Intracellular NOS2 was identified with anti-NOS2 antibody (BD Biosciences, San Jose, CA) followed by anti-rabbit-Alexa Fluor 488 (Invitrogen, Grand Island, NY). To control for nonspecific staining, cells were stained with anti-rabbit Alexa Fluor488 only.

Flow cytometry gating strategy. The flow cytometry gating strategy used to identify macrophages, monocytes, neutrophils, and dendritic cells is shown in Fig. S1 in the supplemental material.

RESULTS

Identification of splenic macrophages by flow cytometry. Previously, hemophagocytes were identified in the bone marrow, spleen, and liver of *S.* Typhimurium-infected Sv129S6 mice, and only rarely was hemophagocytosis noted in control mice (6, 24). A flow-cytometric method was developed to provide a quantitative approach to the analysis of hemophagocytic cells. Since there are no known markers specific for hemophagocytes, putative hemophagocytic cells were identified based on increased cellular DNA content. Hemophagocytes are expected to contain more than 2N DNA based on uptake of leukocytes and erythroblasts, immature red blood cells that accumulate in *S. enterica*-infected mice with the development of regenerative, microcytic anemia (6). Mice were inoculated orally with approximately 10^9 bacteria. At 1, 2, 3, and 4 weeks postinoculation, spleens were harvested. Cells were dissociated and processed for flow cytometry, including staining with anti-CD68 (macrosialin; SR-D1), a monocyte lineage-specific marker (11, 27). Analysis of DAPI staining revealed a typical diploid cell cycle profile with 2N and 4N (termed 2-4N) cells. There was also a smaller population of splenocytes with ≥6N DNA (Fig. 1A). Approximately 4% of $CD68^+$ cells had 6N+ DNA at 2 weeks postinfection (Fig. 1B). Cells with 5N DNA were excluded from analyses to create a buffer zone between 2-4N and 6N+ cells. Cells with 6N+ DNA, which have the potential to be hemophagocytes, were further examined as described below.

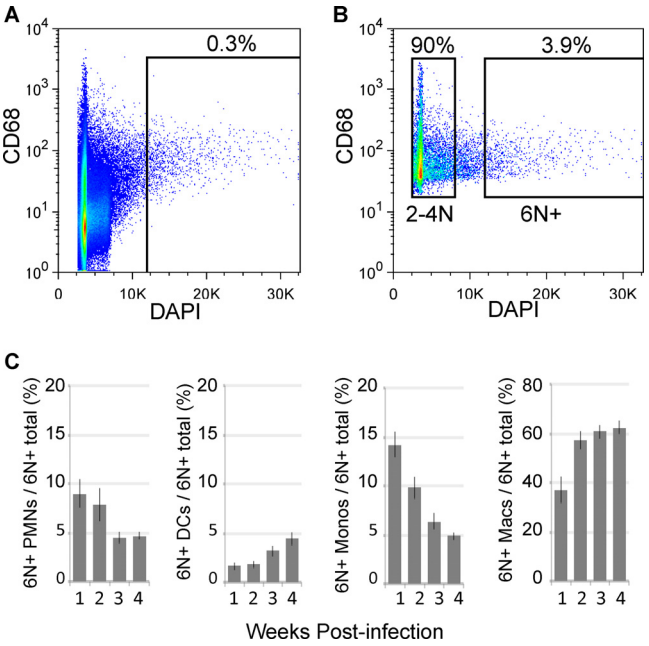

FIG 1 6N+ cells in *Salmonella*-infected mice are predominantly macrophages. Dot plots of total spleen cells (A) and splenic macrophages ($CD68^+$ $CD11c^{low/-}$ Gr-$1^{int/-}$) (B) from a mouse at 2 weeks postinfection (1.0×10^5 CFU per spleen) are shown. (C) The percentage of each type of 6N+ cell over a 4-week infection time course. Neutrophils (PMNs; Gr-1^{high}), dendritic cells (DCs; $CD11c^{high}$), monocytes (Monos; Gr-1^{int} $CD11c^{low/-}$ $CD68^-$), and macrophages (Macs) are shown. Means and SEM from two experiments are shown; $n = 12$ mice at each time point.

Infected mice have 10-fold more 6N+ macrophages than dendritic cells. Human histiocytic diseases can be dominated by hemophagocytes that are macrophages or dendritic cells. This cell type distinction is of clinical significance because it affects diagnosis and treatment (10). Whether macrophages, dendritic cells, or neither predominates in human typhoid fever is not clear because distinguishing markers have not been examined (17, 34). To establish the identity of hemophagocytic cells in murine typhoid fever, spleen cells were dissociated and incubated with antibodies to cell-type-specific markers. Macrophages were identified as $CD68^+$ $CD11c^{low/-}$ (αX integrin, a dendritic cell marker) and Gr-1^{int} (a monocyte and granulocyte/neutrophil marker). Figure S1 in the supplemental material describes the gating procedures.

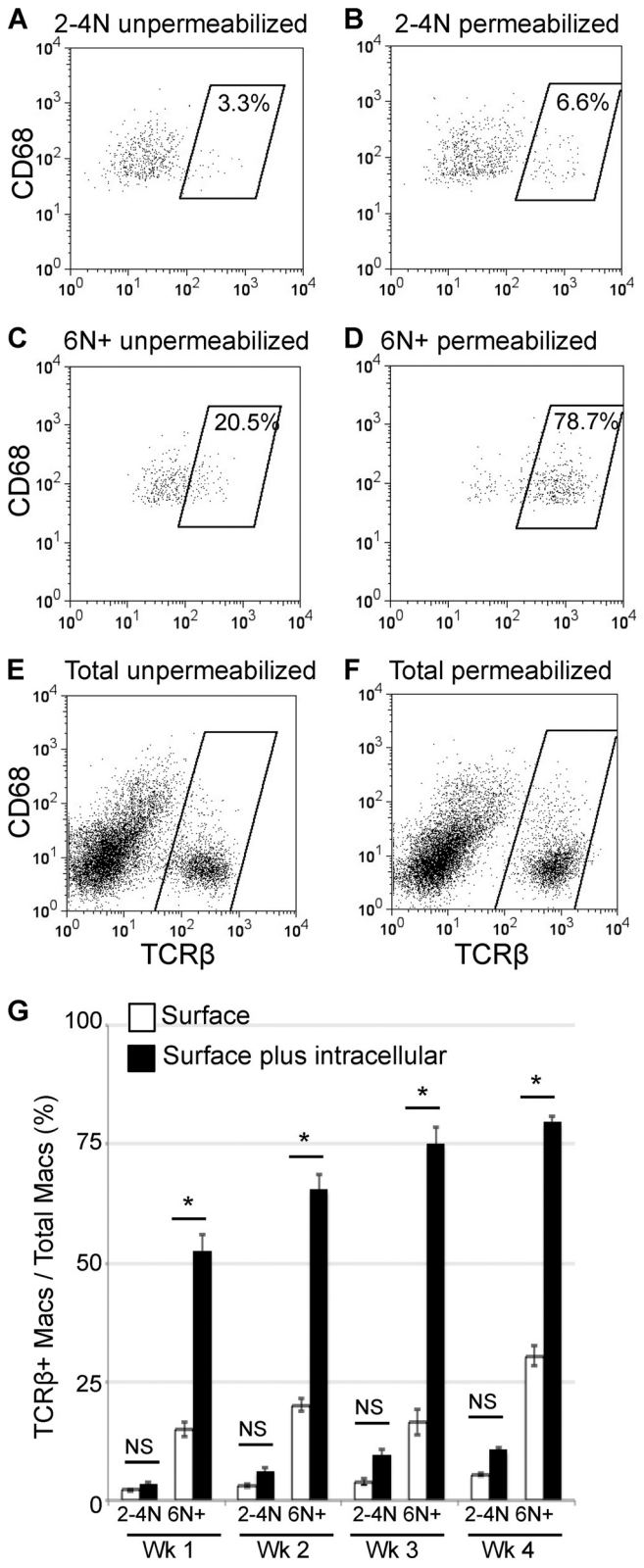

FIG 2 6N+ macrophage population in *Salmonella*-infected mice is enriched for hemophagocytes. (A to D) Gating for TCRβ$^+$ splenic macrophages from a mouse infected for 2 weeks with *S.* Typhimurium (3.8×10^6 CFU per spleen); DNA content and permeabilization treatment are indicated. TCRβ staining of total unpermeabilized (E) and permeabilized (F) spleen cells are shown. (G) Percentage of 2-4N or 6N+ macrophages (Macs; CD68^{high} Gr-1^{int}) positive for surface (white) or surface-plus-intracellular (black) TCRβ. Means and SEM from two experiments are shown. *, $P < 0.0001$ by one-way ANOVA with Tukey's posttest. NS, not significant. $n = 12$, 12, 6, and 12 for weeks 1, 2, 3, and 4, respectively.

Dendritic cells were identified as CD11c^{high}. A third class of professional phagocytes, neutrophils (polymorphonuclear cells, or PMNs), was identified as Gr-1^{high}. Monocytes, immature macrophages, and/or dendritic cells were defined as Gr-1^{int} CD11c$^{low/-}$ CD68$^-$ (15, 28). Over a 4-week infection period, macrophages with 6N+ DNA accumulated to 40 to 60% of all 6N+ cells (Fig. 1C). Dendritic cells and neutrophils accounted for no more than 5 and 10% of 6N+ cells, respectively. However, the number of 6N+ dendritic cells did increase as infection progressed, suggesting these cells play a role in pathogenesis. The percentage of 6N+ monocytes declined as the percentage of 6N+ macrophages increased, consistent with the notion that many infiltrating monocytes matured into tissue macrophages. The difference in the percentage of 6N+ macrophages versus dendritic cells or neutrophils was statistically significant across the infection time course ($P < 0.0005$ by one-way analysis of variance [ANOVA] with a Tukey posttest). These data collectively indicate that murine typhoid fever is dominated by splenic macrophages, not dendritic cells, with increased DNA content.

6N+ macrophages contain T lymphocytes, consistent with hemophagocytosis. In humans and mice, hemophagocytes engulf lymphocytes, among other cell types. To establish whether macrophages with 6N+ DNA contain T cells, an assay comparing surface and intracellular staining to that of a T lymphocyte-specific marker, TCRβ, was developed. Dissociated spleen cells were stained to identify macrophages as described above. Cells were incubated with anti-TCRβ antibody without permeabilization (surface) to identify 6N+ macrophages bound to one or more T cells. Parallel samples were permeabilized and then incubated with anti-TCRβ (surface plus intracellular) to identify 6N+ macrophages bound to and/or containing intracellular T cells (Fig. 2A to D). Gating was performed based on the TCRβ staining pattern observed in the population of total spleen cells (Fig. 2E and F). Over a 4-week infection time course, there was significantly more TCRβ signal in surface-plus-intracellular-stained than in surface-stained 6N+ macrophages (Fig. 2G). Macrophages with 2-4N DNA stained with TCRβ at much lower frequencies, and there were no significant TCRβ staining differences between permeabilized and unpermeabilized samples. The presence of T cells in the majority of 6N+ macrophages in addition to the specificity of CD68 expression for macrophages and not megakaryocytes (21) indicate that megakaryocytes, which have increased levels of DNA, are not included in the 6N+ macrophage populations examined. These observations suggest that infected mice have 6N+ splenic macrophages with engulfed T cells, consistent with hemophagocytosis.

In infected mice, few 6N+ macrophages contain apoptotic cells. A major role of macrophages in health and disease is to phagocytose and catabolize dead or damaged cells. In contrast, hemophagocytic macrophages engulf intact, nonsenescent cells. Cells killed by apoptosis and other mechanisms accumulate breaks in their DNA. To determine whether 6N$^+$ macrophages harbor cells with damaged DNA, the ability of the enzyme terminal deoxynucleotidyl transferase to incorporate the thymidine an-

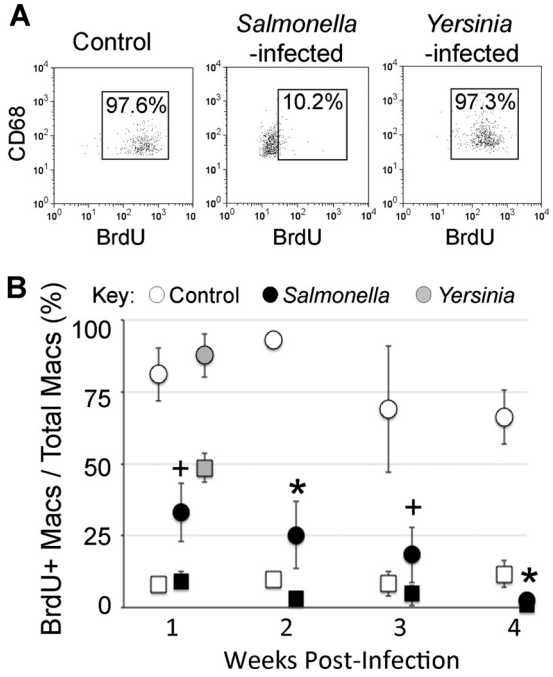

FIG 3 Few 6N+ macrophages in *Salmonella*-infected mice contain broken DNA, a marker of apoptotic or dead cells. (A) Dot plots illustrating gating for $BrdU^+$ cells from control, *Salmonella*-infected (1 week postinfection), and *Yersinia*-infected mice (1 week postinfection). (B) The percentage of 2-4N (squares) and 6N+ (circles) $CD68^+$ cells containing broken DNA ($BrdU^+$) in control (white), *Salmonella*-infected (black), and *Yersinia*-infected (gray; at 1 week) mice. Means and SEM from two experiments are shown. $n = 3$ to 4 at each time point for control mice, $n = 12$ at each time point for *Salmonella*-infected mice, and $n = 9$ for *Yersinia*-infected mice. *P* values compare each 6N+ data point to the corresponding control mice. *, $P < 0.005$; +, $P < 0.05$; each by one-way ANOVA with Tukey's posttest. More than 75% of $CD68^+$ cells are macrophages ($CD68^+$ $CD11c^{low/-}$ Gr-$1^{int/-}$).

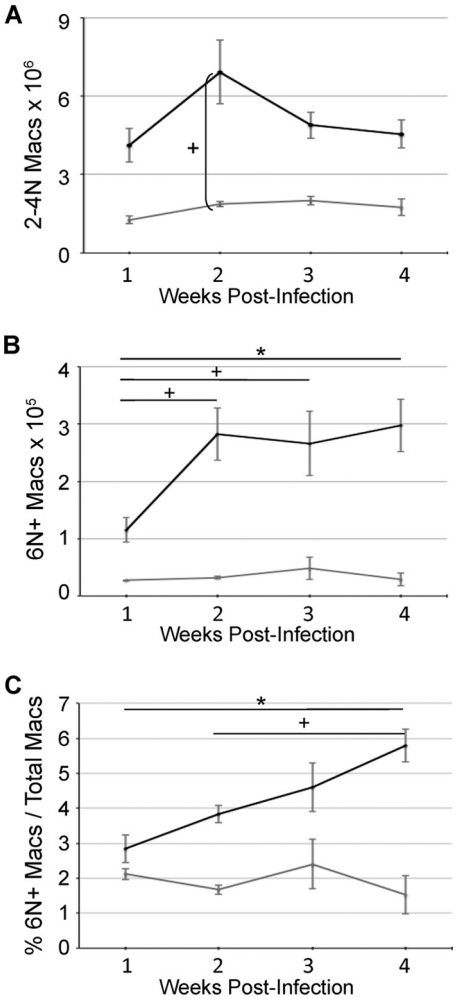

FIG 4 Splenic hemophagocytic macrophages accumulate and are maintained upon infection. Horizontal axes represent a 4-week infection period. (A) Number of 2-4N macrophages (Macs; $CD68^+$ $CD11c^{low/-}$ Gr-$1^{int/-}$) per spleen. (B) Number of 6N+ macrophages per spleen. (C) Percentage of 6N+ macrophages per spleen. Means and SEM from two experiments. $n = 11$ to 12 at each time point for infected mice (black) and 3 to 4 for control mice (gray). *, $P < 0.001$; +, $P < 0.05$; each by one-way ANOVA with Tukey's posttest.

alog BrdU into the DNA of freshly harvested splenic macrophages was examined. $BrdU^+$ cells were identified by flow cytometry using a fluorescently labeled anti-BrdU antibody (Fig. 3A). Approximately 70 to 90% of 6N+ $CD68^+$ cells from control mice were $BrdU^+$, indicating that these histiocytes were either apoptotic or contain apoptotic cells (Fig. 3B). The percentage of 6N+ histiocytes that were $BrdU^+$ declined from an average of 33% at 1 week to less than 3% by 4 weeks, consistent with engulfment of nonapoptotic cells. No more than 12% of 2-4N histiocytes in either infected or uninfected mice were $BrdU^+$ over the 4-week period. In these experiments, 77 to 96% of 6N+ $CD68^+$ cells were macrophages ($CD68^+$ $CD11c^{low/-}$ Gr-1^{int}) in all mice at all times examined. These observations make two important points. First, in infected mice, 6N+ histiocytes, most of which are macrophages, are distinct from those in control mice. Second, 6N+ splenic histiocytes in infected mice are enriched for hemophagocytes.

6N+ macrophages in *Yersinia*-infected mice contain apoptotic cells. *Yersinia* and *Salmonella* are both in the *Enterobacteriaceae* family but shared a last common ancestor approximately 375 million years ago (7). Upon oral inoculation of mice, *Y. pseudotuberculosis* disseminates to and colonizes the spleen and liver, much like *S.* Typhimurium (2). To establish whether $BrdU^-$ 6N+ macrophages accumulate in *Y. pseudotuberculosis*-infected mice, flow cytometry was performed. In these experiments, mice were euthanized at 1-week postinfection, as the Sv129S6 mice had a narrow dosage window within which they could survive infection and remain colonized: doses as high as 2×10^9 CFU resulted in the death of 4/5 mice within a week, whereas doses of less than 0.5×10^9 CFU were cleared within a week. Examination of nine *Y. pseudotuberculosis*-infected mice revealed that 6N+ macrophages in these animals contained $BrdU^+$ DNA, suggesting these macrophages represent apoptotic cells and/or macrophages with engulfed apoptotic cells, not hemophagocytes (Fig. 3A and B). Thus, mice respond differently to *Y. pseudotuberculosis* and *S.* Typhimurium infection, and hemophagocytosis is particular to the latter.

Hemophagocytic macrophages accumulate in *S.* Typhimurium-infected mice. To compare the kinetics of 6N+ and 2-4N macrophage accumulation in the spleen, macrophages were monitored over the first 4 weeks of infection. Relative to control mice, the number of 2-4N splenic macrophages increased and peaked at week 2 (Fig. 4A). The numbers of hemophagocytic macrophages also increased during the first 2 weeks of infection and subsequently were maintained at high levels; as the number of 2-4N

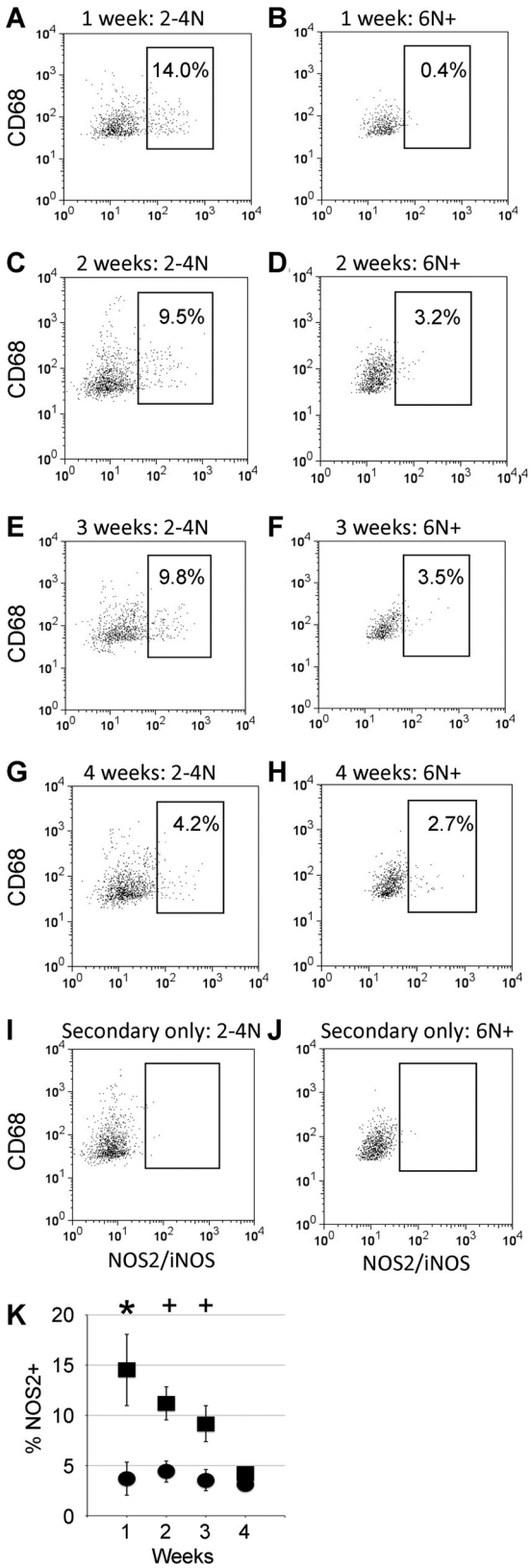

FIG 5 Larger percentages of splenic 2-4N than hemophagocytic macrophages express iNOS/NOS2. (A to H) Percentages of NOS2/iNOS-positive 2-4N and 6N+ macrophages from representative infected mice at 1, 2, 3, and 4 weeks postinfection. (I and J) Staining of 2-4N and 6N+ macrophages from week 2 with the secondary antibody alone. (K) Means and SEM of percentages of NOS2/iNOS-positive 2-4N (square) and 6N+ (circle) macrophages. Data are from two experiments. $n = 12$, 10, 5, and 12 at 1, 2, 3, and 4 weeks postinfection, respectively. *, $P < 0.001$; +, $P < 0.05$; each by Student's two-tailed t test.

macrophages declined, the percentage of hemophagocytic macrophages increased (Fig. 4B and C). In control mice, 6N+ macrophages appear to represent macrophages that have ingested apoptotic cells, consistent with the physiologic role of macrophages in scavenging dead and damaged cells (Fig. 2). 6N+ macrophages in control mice are therefore distinct from those in infected mice but nevertheless provide a point of comparison, with relatively small numbers that change little over time. These data indicate that in infected mice, hemophagocytic macrophages accumulate and persist in the spleen.

A larger percentage of splenic 2-4N than hemophagocytic macrophages express iNOS/NOS2. Macrophages stimulated by the cytokine gamma interferon (IFN-γ) and a Toll-like receptor (TLR) agonist promote a state of classical or M1 activation, which is proinflammatory (22). Infection with *Salmonella* is expected to promote M1 macrophages in the spleen because *Salmonella*-infected mice have high levels of serum IFN-γ, and both lipopolysaccharide (LPS) and flagellin are potent TLR agonists (8). Inducible nitric oxide synthase (NOS2/iNOS) is a marker of M1 macrophages that is important for the production of reactive nitrogen species (22). The mean fluorescence intensity (MFI) of the major population of 6N+ cells was consistently higher than that of 2-4N cells (Fig. 5A versus B, for instance). However, labeling with secondary antibody alone similarly increased the MFI of the major population of 6N+ macrophages, demonstrating little difference in labeling of the major populations of 2-4N and 6N+ cells by the primary iNOS antibody (Fig. 5I versus J). Overall, the analyses revealed that a larger percentage of 2-4N than 6N+ macrophages were iNOS$^+$ at 1, 2, and 3 weeks postinfection (Fig. 5K). These data indicate that 6N+ macrophages are distinct from 2-4N macrophages and may not be classically activated.

Splenic hemophagocytic macrophages express lower levels of proinflammatory markers than 2-4N macrophages. Macrophage expression of additional M1 markers was examined with antibodies to major histocompatibility complex class II (MHC-II) and B7-1 (CD80). The mean fluorescence intensity of MHC-II in hemophagocytic macrophages was approximately 2-fold lower than that of 2-4N macrophages over the course of infection (Fig. 6A and B). Hemophagocytic macrophages also expressed slightly lower levels of CD80 than 2-4N macrophages, particularly at 2 weeks postinfection. Together, these observations provide additional evidence that hemophagocytic macrophages in murine typhoid fever are not classically activated.

Hemophagocytic macrophages express markers consistent with an M2 activation state. Whether hemophagocytic macrophages express markers of an anti-inflammatory or M2 state was examined. Scavenger receptor class B1 (SRB1/CD36) recognizes lipids and can bind pathogens, including *S.* Typhimurium (11). SRB1 expression in macrophages increases upon exposure to microbes such as *S.* Typhimurium (1). In hemophagocytic macrophages, SRB1 was expressed at higher levels than in 2-4N macrophages at 3 and 4 weeks postinfection (Fig. 6A and B). A second marker of M2 macrophages is the C-type lectin mannose receptor (MRC1/CD206) that is involved in endocytosis (11). MRC1 was

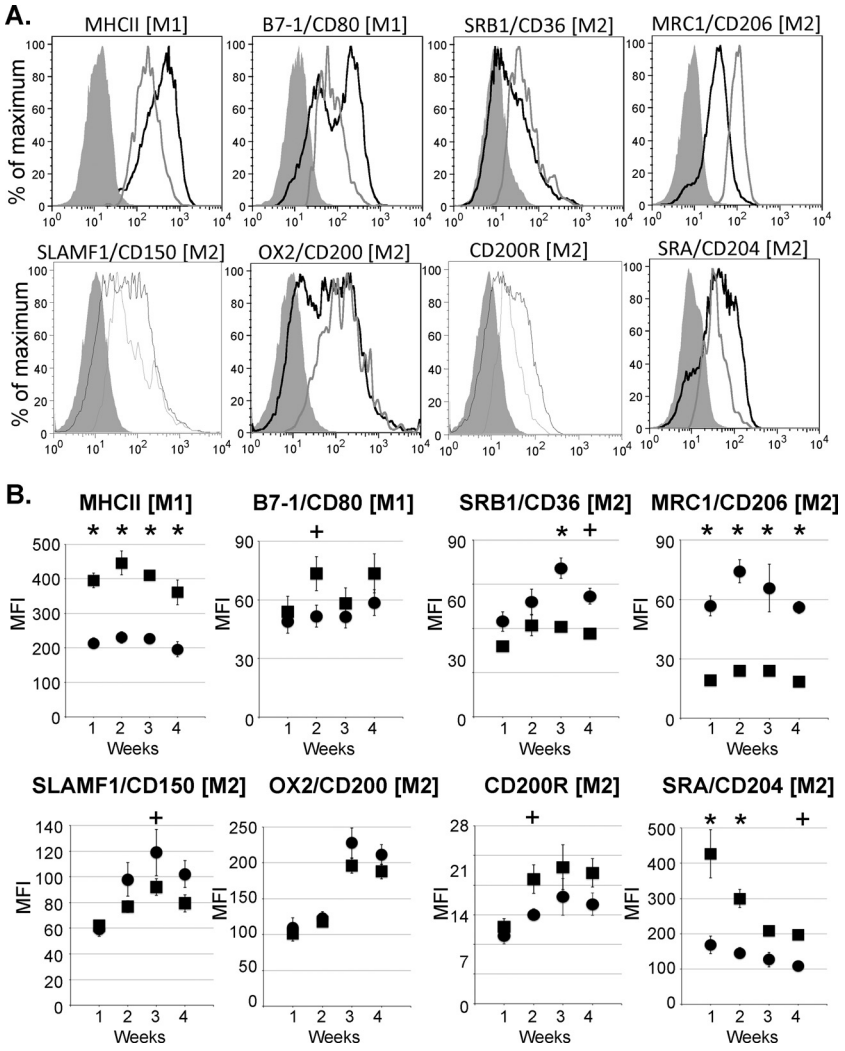

FIG 6 Splenic hemophagocytic macrophages are distinct from 2-4N macrophages and express markers consistent with an M2 phenotype. (A) Histograms from representative mice of 2-4N (black line) and 6N+ (gray line) macrophages stained with the indicated marker and isotype controls (filled). (B) Means and SEM of MFI of 2-4N (square) and 6N+ (circle) macrophages. Data are from two experiments. n = from 11 to 12 mice at each week postinfection. *, $P < 0.001$; +, $P < 0.05$; each by Student's two-tailed t test.

also more highly expressed on hemophagocytic than 2-4N macrophages at all time points postinfection. These data suggest hemophagocytic macrophages have an M2 activation state.

Three additional M2 markers examined were signaling lymphocytic activation molecule family member 1 (SLAMF1/CD150), OX2/CD200, and CD200R. SLAMF1 inhibits the induction of proinflammatory cytokines (29) and is more highly expressed on hemophagocytic than on 2-4N macrophages at 3 weeks postinfection (Fig. 6A and B). CD200 and CD200R represent a receptor-ligand pair expressed on macrophages that is important for reducing macrophage activation during infection with *Neisseria meningitides* (16, 23). Surface expression of the CD200 ligand was slightly but not significantly higher on hemophagocytic macrophages than 2-4N macrophages. Expression of the CD200R was lower on hemophagocytic macrophages, significantly so at 2 weeks postinfection. These observations are consistent with the expression patterns described for CD200 and CD200R in response to *N. meningitides* infection (23) and support an anti-inflammatory phenotype for hemophagocytic macrophages.

Hemophagocytic macrophages express low levels of a marker for apoptotic cell uptake. Scavenger receptor class A type I/II (SRA/MSR1/CD204) is a marker of a subclass of M2 macrophages involved in the clearance of apoptotic cells (9, 26). Flow cytometry identified hemophagocytic macrophages that expressed significantly lower levels of SRA than 2-4N macrophages at 1, 2, and 4 weeks postinfection, suggesting hemophagocytes do not participate in the engulfment of apoptotic cells (Fig. 6A and B). These data are consistent with the observation of few apoptotic cells in hemophagocytic macrophages (Fig. 2) and further underscore that hemophagocytic macrophages have properties distinct from those of macrophages with ingested apoptotic cells.

DISCUSSION

Light microscopy demonstrated the presence of hemophagocytic histiocytes containing erythroblasts and lymphocytes in a natural host-pathogen infection, murine typhoid fever (6, 24). We therefore characterized hemophagocytes in Sv129S6 mouse spleens by flow cytometry. Hemophagocytic macrophages were identified

based on the expression of macrophage-specific markers, increased DNA that is intact, and enrichment for intracellular T cells. The 6N+ macrophage population also expressed low levels of SRA/CD204, suggesting these macrophages do not contain apoptotic cells (9, 26). Thus, 6N+ macrophages identified by flow cytometry are enriched for hemophagocytes.

Hemophagocytic macrophages outnumbered hemophagocytic dendritic cells 10 to 1 in Sv129S6 mice with murine typhoid fever. This has potential clinical importance, as human hemophagocytic diseases are diagnosed and treated based on whether they are dominated by dendritic cells (e.g., Langerhans cell histiocytosis) or macrophages (e.g., hemophagocytic lymphohistiocytosis [HLH], or MAS) (10, 13). Whether hemophagocytosis in typhoid fever patients is macrophage or dendritic cell related is not clear (17, 34). Given that markers distinguishing between human macrophages and dendritic cells now exist, this issue could be revisited in bone marrow biopsy samples from confirmed typhoid patients.

Both human and murine hemophagocytic macrophages can express markers consistent with an M2 phenotype. In patients with sepsis or MAS, hemophagocytic macrophages express high levels of CD163, a hemoglobin scavenger receptor and M2 marker (31, 32). In murine typhoid fever, hemophagocytic macrophages with an M2 activation state accumulate within 1 week of inoculation. However, not all M2 markers are highly expressed on hemophagocytic macrophages in mice, likely reflecting the multiple different classes of M2 macrophages that have been described (20, 22). For instance, macrophages that engulf apoptotic cells are considered to have an M2 activation state and can express SRA/CD204 (36). Hemophagocytic macrophages express lower levels of SRA/CD204 than 2-4N macrophages, suggesting that 6N+ macrophages represent a different kind of M2 macrophage that does not remove apoptotic cells. Which, if any, M2 subclass hemophagocytic macrophages in *S.* Typhimurium-infected mice represent is important to determine and will require more extensive marker analysis.

How hemophagocytic macrophages acquire and/or maintain an M2 phenotype is not known. During the first few days of infection with *Salmonella*, mice develop elevated serum IFN-γ but serum tumor necrosis factor alpha (TNF-α) remains low, at less than 100 pg/ml (8). High expression of NOS2, MHC-II, and CD80 by 2-4N macrophages in mice infected with *S.* Typhimurium indicates that these cells become classically activated, presumably via exposure to IFN-γ and expression of TNF-α. The M2 phenotype of hemophagocytic macrophages suggests that these cells do not produce sufficient TNF-α to become classically activated. TNF-α production is limited in mice and macrophages infected with *N. meningitides* by CD200. Infection with either *S.* Typhimurium (Fig. 6) or *N. meningitides* increases macrophage expression of CD200 while decreasing expression of CD200R (23). These data suggest that CD200-CD200R reduces hemophagocytic macrophage activation. It will be of interest in the future to determine which, if any, of these mechanisms support the establishment and/or maintenance of an M2 activation state in hemophagocytes.

The apparent absence of hemophagocytic macrophages in mice infected with *Y. pseudotuberculosis* suggests that the host responds differently to infection with different microbes, even though both *Y. pseudotuberculosis* and *S.* Typhimurium colonize the same tissues and reside within macrophages in the spleen (4, 12). One clue is that a larger percentage of 2-4N macrophages from *Y. pseudotuberculosis*-infected mice have damaged DNA compared to 2-4N macrophages from *S.* Typhimurium or control mice ($P < 0.005$ and 0.05, respectively, by one-way ANOVA with a Tukey posttest) (Fig. 3). These data suggest that there is more apoptosis upon infection with *Y. pseudotuberculosis* than with *S.* Typhimurium, resulting in macrophage clearance of apoptotic cells in the former. In other words, hemophagocytosis may be a consequence of inflammation in the absence of increased apoptosis.

What is the larger significance of an M2 activation phenotype for hemophagocytic macrophages and *Salmonella* infection? One hypothesis is that these cells are part of a host effort to downregulate acute inflammation. Inflammation is necessary to fight infection but can also cause lethal damage. If inflammation is suppressed prior to pathogen clearance, damage is avoided but persistent infection with the potential for relapse may result. Whether and how hemophagocytic macrophages influence the balance between containing bacteria and limiting damage is complicated by the observation that *Salmonella* resides within hemophagocytic macrophages of the liver based on confocal microscopy (24). We do not know yet whether hemophagocytic macrophages identified by flow cytometry from the spleen also contain the bacteria. Additional crucial issues include (i) whether *Salmonella* is present in other cell types and (ii) in which cell type(s) are the bacteria metabolically active. Thus, the role of hemophagocytosis in *Salmonella* infection remains an interesting area of research.

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M.W.M. designed and performed research, analyzed data, and helped write the paper. S.M.M. performed research, assisted with data analysis, and reviewed the paper. C.S.D. designed research methods, analyzed data, and wrote the paper.

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