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Neutrophils from Both Susceptible and Resistant Mice Efficiently Kill Opsonized *Listeria monocytogenes*

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ABSTRACT Inbred mouse strains differ in their susceptibility to infection with the facultative intracellular bacterium *Listeria monocytogenes*, largely due to delayed or deficient innate immune responses. Previous antibody depletion studies suggested that neutrophils (polymorphonuclear leukocytes [PMN]) were particularly important for clearance in the liver, but the ability of PMN from susceptible and resistant mice to directly kill *L. monocytogenes* has not been examined. In this study, we showed that PMN infiltrated the livers of BALB/c/By/J (BALB/c) and C57BL/6 (B6) mice in similar numbers and that both cell types readily migrated toward leukotriene B4 in an *in vitro* chemotaxis assay. However, CFU burdens in the liver were significantly higher in BALB/c mice than in other strains, suggesting that PMN in the BALB/c liver might not be able to clear *L. monocytogenes* as efficiently as B6 PMN. Unprimed PMN harvested from either BALB/c or B6 bone marrow killed *L. monocytogenes* directly *ex vivo*, and pretreatment with autologous serum significantly enhanced killing efficiency for both. *L. monocytogenes* were internalized within 10 min and rapidly triggered intracellular production of reactive oxygen species in a dose-dependent manner. However, PMN from gp91^{phox}-deficient mice also readily killed *L. monocytogenes*, which suggested that nonoxidative killing mechanisms may be sufficient for bacterial clearance. Together, these results indicate that there is not an intrinsic defect in the ability of PMN from susceptible BALB/c mice to kill *L. monocytogenes* and further suggest that if PMN function is impaired in BALB/c mice, it is likely due to locally produced modulating factors present in the liver during infection.

KEYWORDS BALB/cBy/J mice, C57BL/6 mice, innate immunity, reactive oxygen species, liver

*L*isteria monocytogenes is a facultative intracellular bacterium that causes foodborne disease in humans. In mouse models of listeriosis, orally transmitted *L. monocytogenes* establishes infection in the gut and then spreads systemically to the spleen, liver, and gallbladder (1, 2). Susceptibility to listeriosis varies among inbred mouse strains; for example, compared to what is seen in C57BL/6 (B6) or C57BL/10 mice, bacterial burdens rise to significantly higher levels and clearance is delayed in BALB/c mice (2–4). This difference has been attributed primarily to facets of innate immunity, including differential expression of STAT4 and reduced or delayed secretion of tumor necrosis factor alpha (TNF-α), interleukin-12 (IL-12), and gamma interferon (IFN-γ) (5–8).

One mechanism for innate clearance of *L. monocytogenes* is direct killing by neutrophils (polymorphonuclear leukocytes [PMN]). Early work to deplete PMN from mice relied on an antibody (clone RB6-8C5) that was subsequently shown to bind both PMN and monocytes (9–13). More recent depletion strategies designed to avoid this issue yielded conflicting results about the importance of PMN for clearance from the liver (14, 15). PMN in the liver were found either to kill bacteria released from apoptotic hepatocytes or perhaps to directly lyse infected hepatocytes, releasing the bacteria
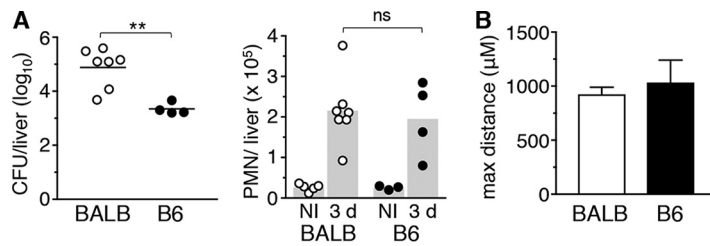
FIG 1 Neutrophil recruitment to the liver following foodborne infection is similar in B6 and BALB/c mice. (A) Mice were fed 3×10^8 CFU of *L. monocytogenes* SD2000. At 3 dpi, livers were perfused and total CFU was determined. The total number of Ly6G^{hi} Ly6C$^+$ PMN found in the nonparenchymal fraction after tissue digestion in uninfected (NI) or infected (3 d) mice was also determined. Pooled results from three independent experiments are shown. (B) Migration of gradient-enriched PMN toward 100 nM leukotriene B4 was monitored using an under-agarose assay. Representative results from one of three independent experiments are shown ($n = 4$ technical replicates per group).

from a protective niche (16–18). Despite PMN recruitment to the liver, CFU burdens in BALB/c mice increased steadily for approximately 5 days; in contrast, exponential growth of *L. monocytogenes* was restricted in B6 livers and the number of CFU began to fall after approximately 3 days (2, 3). This suggests that BALB/c PMN might be less efficient at killing *L. monocytogenes* than B6 PMN.

PMN make up the majority of immune cells in human blood but account for only about 15% of circulating immune cells in the mouse (19, 20). Since human blood is readily available in larger quantities than murine blood, human PMN are most commonly used for *in vitro* assays that study interactions of PMN with bacteria. Murine PMN lack defensins and have a low affinity for the prototypical formylated peptide formyl-methionyl-leucyl phenylalanine (fMLF), but they do possess a strong affinity for formylated peptides derived from *L. monocytogenes* and *Staphylococcus aureus* (21–23). Differences in relative speed and migration distance have also been noted when murine and human PMN were compared in chemotaxis assays (24). The difficulty in obtaining large numbers of PMN from mice and the potential for functional differences with human cells have resulted in a lack of studies using murine PMN. However, PMN isolated from human peripheral blood cannot be used to evaluate how PMN contribute to susceptibility or resistance in mouse models of infection.

It has been estimated that mature PMN remain in the bone marrow of mice for up to 2 days before being released into circulation (25). Boxio et al. first showed that mouse bone marrow is an abundant source of morphologically mature PMN and that these cells release both primary and secondary granules upon stimulation (26). Swamydas and Lionakis further refined the enrichment protocol and showed that PMN harvested from bone marrow could be used for adoptive transfer (27). In this study, we used PMN enriched from the bone marrow of naive or infected BALB/c and B6 mice and compared the abilities of the cells to kill *L. monocytogenes* directly *ex vivo*. We demonstrate that murine neutrophils efficiently kill serum-opsonized *L. monocytogenes* using both oxidative and nonoxidative mechanisms. We concluded that there were no intrinsic differences in the capacity of PMN from susceptible BALB/c and resistant B6 mice to directly kill *L. monocytogenes*.

RESULTS

Hepatic PMN infiltration after foodborne infection is similar in BALB/c and B6 mice. Using the foodborne model of listeriosis in mice, *L. monocytogenes* colonizes the gut tissue for 24 to 48 h and then spreads systemically to the spleen and liver (2, 28). Differences in host susceptibility to infection can readily be observed in the liver, where the number of CFU in susceptible BALB/c mice is significantly greater than in the more resistant B6 mice (Fig. 1A) (2). Neutrophil-rich hepatic abscesses containing *L. monocytogenes* were observed in both BALB/c and B6 mice 24 h after intravenous infection (29). To verify that PMN were recruited to the liver parenchyma after foodborne infection, livers were harvested after portal vein perfusion to remove blood, and Ly6G^{hi}
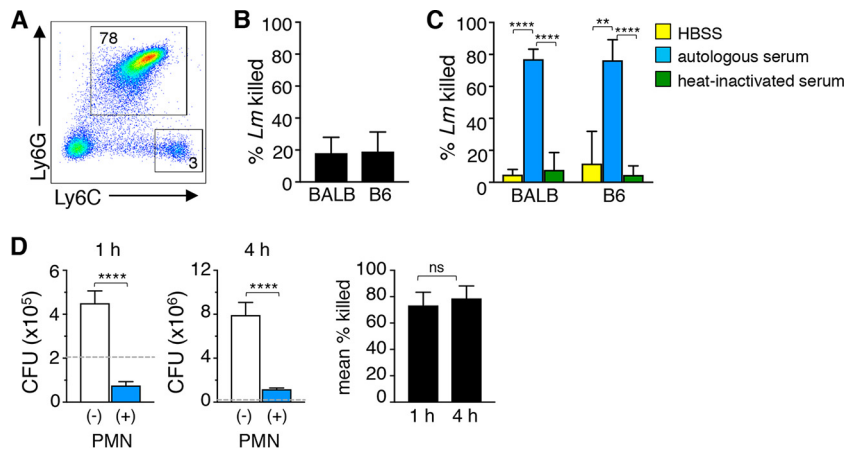
FIG 2 Murine neutrophils kill *L. monocytogenes* efficiently in the presence of serum. Gradient-enriched PMN were used to perform *in vitro* killing assays. (A) A representative dot plot shows the composition of a typical gradient-enriched bone marrow prep; live- and single-cell gates were applied. (B) PMN (*n* = 3 technical replicates) were incubated with *L. monocytogenes* at an MOI of 0.05 for 90 min. Pooled results from 2 independent experiments are shown. (C) *L. monocytogenes* bacteria were pretreated as indicated and added to PMN (MOI = 0.1) for 60 min. Representative results from one of three independent experiments are shown. (D) Serum-opsonized *L. monocytogenes* (MOI = 2) was added to BALB/c PMN. The absolute number of CFU remaining in the wells at either 1 h or 4 h for a representative experiment is shown in the two graphs on the left. Dashed lines indicate the number of CFU at *t* = 0. The mean percentage killed at each time point (± SD) for three independent experiments is shown on the right. ns, not significant.

cells were identified by flow cytometry. At 3 days postinfection (dpi), there was a 5- to 10-fold increase in the number of PMN in the liver, with no significant difference observed between BALB/c and B6 mice (Fig. 1A). Thus, a lack of PMN infiltration did not explain the greater CFU burden in the BALB/c liver.

After reaching a tissue such as the liver, PMN must further sense and then migrate toward specific chemoattractants, a process that was recently shown to create a "swarm" at infectious foci (30). To test whether BALB/c cells had a chemotaxis defect, we analyzed the ability of gradient-enriched PMN to migrate toward specific signals using an under-agarose assay. We first tested *L. monocytogenes*-derived formylated peptides, which were previously shown by Liu et al. to induce chemotaxis from mouse PMN in a Boyden chamber assay (31). A combination of two formylated peptides (1 μM fMIVTLF plus 1 μM fMIGWII; Bio-Synthesis, Inc.) did not induce migration in the under-agar assay, despite triggering a burst of intracellular ROS when applied to either BALB/c or B6 PMN (data not shown). However, leukotriene B4, a lipid-derived chemotactic signal produced by PMN (32), did induce migration of gradient-enriched murine PMN. The maximum distance traveled in 3 h was approximately 900 μm for both BALB/c and B6 PMN (Fig. 1B). Together, these data indicated that recruitment of circulating PMN and chemotaxis toward PMN-derived stimuli was similar in susceptible BALB/c mice and resistant B6 mice.

Unprimed bone marrow neutrophils rapidly kill *L. monocytogenes* in the presence of autologous serum. Although there was no difference in PMN infiltration, increased bacterial growth in BALB/c mice might also be due to an intrinsic defect in the ability of BALB/c PMN to kill *L. monocytogenes*. To test this, we developed an *in vitro* killing assay using PMN isolated from the bone marrow of uninfected mice. PMN were enriched using gradient centrifugation, a method that typically yielded a 65 to 80% pure population of Ly6G^{hi} neutrophils (Fig. 2A). The remaining cells were Ly6C^{hi} monocytes (1 to 5%), CD19$^+$ B cells (10 to 20%), and a small number of CD11b$^+$ CD64$^-$ cells (data not shown). The enriched PMN were transferred to a 96-well flat-bottom plate and incubated with *L. monocytogenes* at a low multiplicity of infection (MOI) for 90 min. As shown in Fig. 2B, there was only a 20% decrease in detectable CFU within this time frame using PMN from either mouse strain.
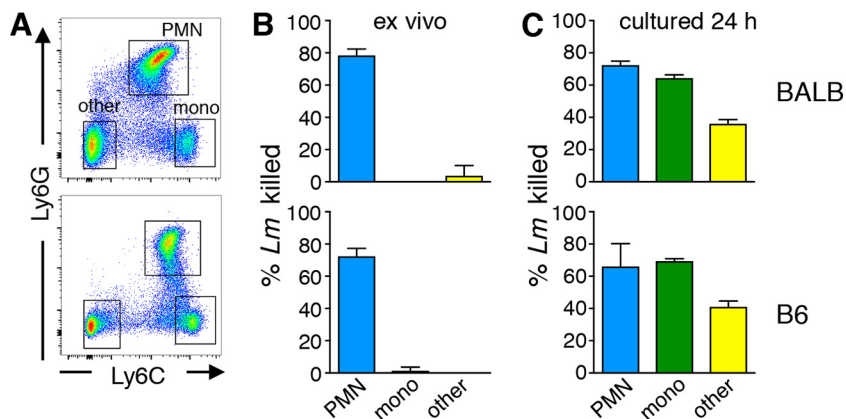
FIG 3 Sorted murine neutrophils efficiently kill *L. monocytogenes*. (A) Representative plots depict the three populations of cells sorted from marrow of BALB/c (top row) and B6 (bottom row) mice. (B) Sorted cells were plated in medium for 1 h, and then serum-opsonized *L. monocytogenes* was added (MOI = 0.1); killing efficiency was assessed after 50 min. (C) Sorted cells were cultured overnight at 37°C in 7% CO_2. The following day, a killing assay was performed as described above. Results from one of two independent experiments with 3 technical replicates per group are shown.

Bortolussi et al. previously showed that *L. monocytogenes* uptake by human PMN was greatly enhanced when serum was present (33). To find out if killing efficiency could be improved by exposure to serum proteins, we pretreated late-stationary-phase bacteria with 10% autologous mouse serum for 30 min prior to the addition of PMN. As shown in Fig. 2C, serum pretreatment significantly increased the killing rate within 1 h for both BALB/c and B6 PMN. In contrast, pretreatment with heat-inactivated serum did not enhance killing of *L. monocytogenes*. Thus, a heat-labile component found in naive serum, most likely complement, contributed significantly to the ability of PMN to kill *L. monocytogenes in vitro*. Accordingly, serum pretreatment was used for all subsequent killing assays.

In the killing assays described above, the percentage of *L. monocytogenes* cells killed was calculated by comparing the number of CFU recovered in the presence of PMN to the number of CFU found in assay wells that contained only bacteria. Incubation of *L. monocytogenes* in tissue culture medium for 1 h resulted in an approximate doubling of the bacteria (Fig. 2D), a pattern that was consistently observed in all assays. The number of CFU recovered after the addition of gradient-enriched PMN was significantly less than the initial inoculum (Fig. 2D, dashed lines), demonstrating that the PMN were actively killing bacteria within 1 h rather than simply inhibiting growth. However, given that the PMN outnumbered the bacteria in these assays up to 10-fold, it was surprising that so many viable *L. monocytogenes* cells remained in each well. To find out if a longer incubation time would allow the PMN to completely clear the inoculum, the killing assay was extended to 4 h. *L. monocytogenes* incubated in tissue culture medium alone increased more than 30-fold during this time frame (Fig. 2D). When PMN were added, the number of *L. monocytogenes* bacteria was 5-fold higher than the initial inoculum, but this number represented an approximate 80% killing rate compared to bacterial growth without PMN. Thus, prolonged incubation time did not increase the efficiency of killing, and extending the incubation period just allowed any bacteria that evaded contact with PMN to replicate exponentially.

Sorted bone marrow PMN maintain viability and killing capacity after *in vitro* culture. Although gradient centrifugation provided a substantially enriched PMN population to study, it was unknown whether any of the killing observed could be ascribed to the contaminating cells, which typically made up 20 to 35% of the enriched population. To investigate this, three populations of cells were sorted from BALB/c and B6 bone marrow: Ly6G^{hi} Ly6C$^+$ PMN, Ly6C^{hi} Ly6G$^-$ monocytes, and the residual Ly6G$^-$ Ly6C$^-$ cells (Fig. 3A). Each population was plated separately and incubated with *L. monocytogenes* for 1 h. As shown in Fig. 3B, sorted PMN from both BALB/c and B6 mice
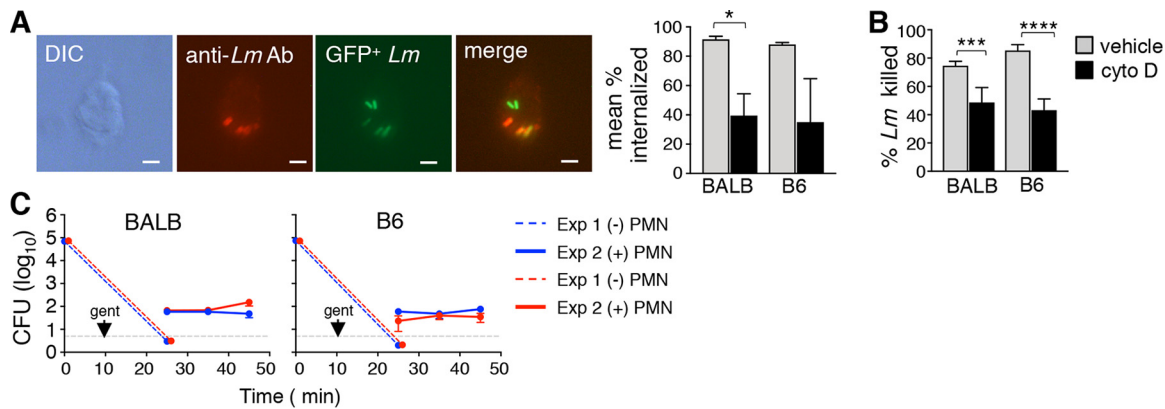
FIG 4 Murine neutrophils rapidly internalize *L. monocytogenes in vitro*. (A) Serum-opsonized *L. monocytogenes* bacteria constitutively expressing GFP (*L. monocytogenes* SD2710) were added to sorted PMN (MOI = 1) that were pretreated for 45 min with either 20 μM cytochalasin D (cyto D) or DMSO (vehicle). Cell-associated *L. monocytogenes* bacteria were visualized by differential in/out staining 10 min postinfection. Representative images show intracellular (green) and extracellular (yellow) bacteria (bar, 2 μM), and the mean percentage of internalized bacteria in three independent experiments is shown. (B) A representative killing assay was performed at 50 min for cells treated as described for panel A; bars indicate means ± standard deviations (SD). (C) Gentamicin protection assay performed with serum-opsonized *L. monocytogenes* and gradient-enriched PMN (n = 3 technical replicates) and incubated for 10 min before addition of gentamicin. Gentamicin was added at t = 10 min as indicated by the arrowheads. Data from two independent experiments (Exp 1, Exp 2) are shown.

killed nearly 80% of the bacteria. Neither monocytes nor the residual population of primarily B cells were capable of killing the bacteria directly *ex vivo*. These results indicated that murine bone marrow PMN could be sorted without deleterious effects and suggested that the killing observed with gradient-enriched cells was attributable only to the PMN.

To assess the functional *ex vivo* life span of murine bone marrow PMN, sorted cells were cultured overnight and inoculated with *L. monocytogenes* the following day. As shown in Fig. 3C, PMN remained capable of killing 60 to 70% of the inoculum after 24 h of *in vitro* culture. However, in contrast to what was observed directly *ex vivo*, Ly6C^{hi} monocytes also killed approximately 60% of bacteria. Surprisingly, even the other cells, which were comprised mainly of CD19^{+} B cells, killed 30 to 40% of *L. monocytogenes* cells (Fig. 3C). Therefore, murine PMN are viable and retain efficient killing ability for at least 24 h after isolation from the bone marrow. However, these data indicate that only highly purified sorted PMN should be used for experiments involving cells maintained in culture for more than a few hours.

Bone marrow PMN rapidly internalize and kill *L. monocytogenes* intracellularly. Human PMN are known to phagocytose particulate matter within seconds, followed by rapid closure of the phagosome, which then fuses with granules containing an array of antibacterial compounds (34). Extracellular killing mechanisms such as neutrophil extracellular traps have also been described (35). To assess the importance of bacterial internalization for *in vitro* killing of *L. monocytogenes*, sorted PMN were pretreated with cytochalasin D, an inhibitor of actin polymerization. *L. monocytogenes* bacteria constitutively expressing green fluorescent protein (GFP) were added, and differential "in/out" staining was performed 10 min later. Nonpermeabilized cells were stained with Texas Red-conjugated polyclonal anti-*L. monocytogenes* antibodies such that cell-associated extracellular bacteria appeared yellow (Fig. 4A). Bacteria that were internalized by the PMN, and thus not accessible to the antibodies, appeared green. As shown in Fig. 4A, approximately 90% of the cell-associated *L. monocytogenes* cells were intracellular 10 min after incubation with either BALB/c or B6 PMN. Pretreatment with cytochalasin D decreased the percentage of intracellular bacteria but did not completely block uptake. Killing assays performed simultaneously also showed a significant reduction in the percentage of bacteria killed when cytochalasin D was used (Fig. 4B). These results indicated that phagocytosis contributes to the ability of mouse bone marrow PMN to efficiently kill serum-opsonized *L. monocytogenes*.
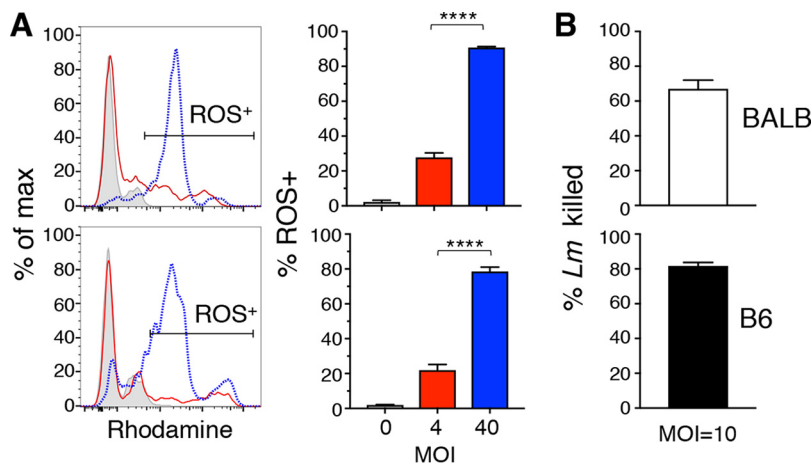
FIG 5 *L. monocytogenes* induces a dose-dependent ROS burst in murine neutrophils. Gradient-enriched neutrophils suspended in HBSS were incubated with dihydrorhodamine 123 prior to the addition of *L. monocytogenes* at MOIs of 4 (red) and 40 (blue); mock-infected cells are shown in gray. (A) Representative histograms for cells harvested 30 min after addition of bacteria and total ROS$^+$ Ly6G^{hi} Ly6C$^+$ PMN are shown. (B) Gradient-enriched PMN were used for an *in vitro* killing assay (MOI = 10). Killing efficiency was assessed after 50 min. Representative results from one of three experiments using 3 to 5 technical replicates per treatment are shown. Bars indicate means ± SD.

To investigate the possibility that *L. monocytogenes* might be efficiently internalized by murine PMN but not killed within the short time period of these assays, a gentamicin protection assay was performed to quantify intracellular bacteria. *L. monocytogenes* was added to gradient-purified PMN, and gentamicin was added 10 min later. As shown in Fig. 4C, 10 μg/ml gentamicin was sufficient to kill 100% of the 1×10^5 CFU added to each well in the absence of PMN. At 25 min, 50 to 200 gentamicin-resistant CFU were detected per well (0.05 to 0.2% of the initial inoculum). The number of intracellular *L. monocytogenes* bacteria did not change at 35 or 45 min postinfection (Fig. 4C). These data suggested that a small number of *L. monocytogenes* cells may be able to avoid killing and survive intracellularly. However, the negligible number of gentamicin-resistant bacteria were not likely to influence the percent killed calculations in the *in vitro* killing assays.

***L. monocytogenes* induces a respiratory burst in unprimed murine PMN.** One of the ways PMN can kill bacteria is though targeted release of reactive oxygen species (ROS). To measure the induction of ROS in PMN exposed to *L. monocytogenes*, dihydrorhodamine 123 (DHR) fluorescence was used. DHR diffuses across the cell membrane and fluoresces when oxidized to rhodamine by either peroxynitrite, a product of nitric oxide and superoxide, or hypochlorous acid (36). The short incubation period of the assays described here was not likely to activate inducible nitric oxide synthase (iNOS) (37, 38); therefore, in this system, DHR served primarily as a measure of the amount of hypochlorous acid produced by the PMN.

Gradient-enriched PMN from BALB/c and B6 mice were incubated in medium for 1 h and then suspended in buffer prior to the addition of serum-opsonized *L. monocytogenes*. As shown in Fig. 5A, when the bacteria were added at a low MOI, less than 25% of the PMN produced ROS within 30 min. When 10-fold-more bacteria were added, the number of ROS-positive cells increased significantly for both BALB/c and B6 PMN. Thus, unprimed PMN from both mouse strains rapidly responded to the presence of *L. monocytogenes* by producing ROS in a dose-responsive manner. Although increasing the MOI resulted in a more robust respiratory burst, the killing efficiency remained at 60 to 80% (Fig. 5B), as was observed previously for assays using lower MOIs (compare Fig. 5B to Fig. 2C, 3B, and 4B). Together, these observations suggested that ROS might not be required for efficient killing during the *in vitro* assay.

Murine PMN use both oxidative and nonoxidative mechanisms to kill *L. monocytogenes*. To specifically assess the importance of ROS for killing of *L. monocytogenes*,
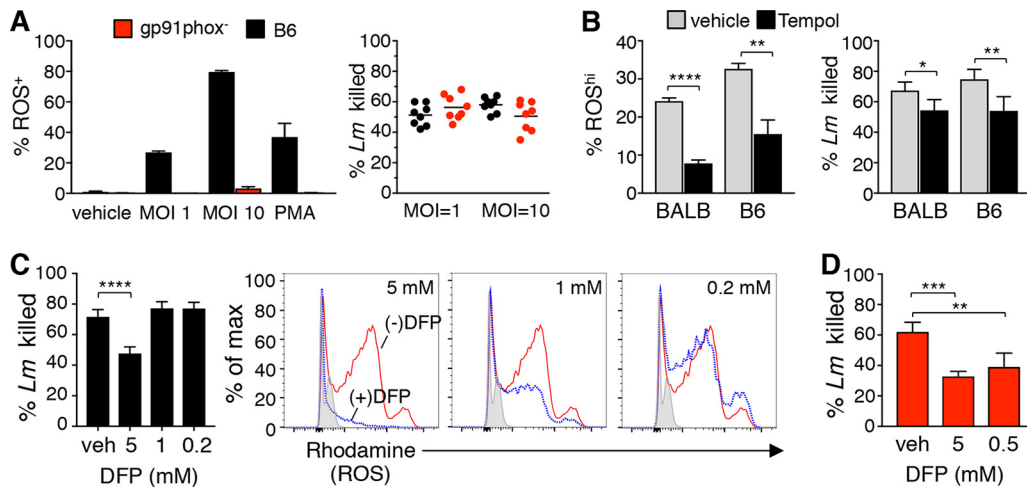
FIG 6 ROS is not required for efficient in vitro killing of L. monocytogenes. (A) Gradient-enriched PMN from gp91$^{phox-/-}$ and B6 mice were loaded with DHR prior to addition of either serum-opsonized L. monocytogenes or 20 nM PMA. ROS levels were determined at 30 min, and a killing assay was performed at 50 min. Pooled data from two independent experiments are shown; horizontal bars indicate means ± SD. (B) Gradient-enriched PMN were incubated with 50 μM Tempol or vehicle for 90 min prior to addition of L. monocytogenes, and the percentage of ROS^{hi} neutrophils and the killing efficiency for these cells were determined. Data from one of three independent experiments are shown; bars indicate means ± SD. (C) Gradient-enriched BALB/c PMN were incubated with DFP for 30 min at the indicated concentrations prior to addition of L. monocytogenes (MOI = 10). ROS activity was determined 30 min later, and the percent killed was determined 50 min after addition of bacteria (n = 5). Representative results from one of two independent experiments are shown. Solid gray histogram indicates uninfected cells. (D) Gradient-enriched PMN from gp91$^{phox-/-}$ males were treated with DFP at the indicated concentrations or vehicle (veh) for 30 min prior to addition of L. monocytogenes (MOI = 10) for an in vitro killing assay. Bars indicate means for 6 replicates from one of two independent experiments.

PMN from gp91^{phox}-deficient mice were compared to wild-type B6 PMN. The gp91^{phox}-deficient mice lack a functional allele for the large gp91 subunit of NADPH oxidase; thus, the enzyme cannot assemble (39). As shown previously, there was a dose-dependent response for wild-type B6 PMN, with an MOI of 10 inducing more ROS-positive cells than even phorbol myristate acetate (PMA) treatment (Fig. 6A). PMN from gp91^{phox}-deficient mice produced no detectable ROS at low MOI or after treatment with PMA, but 2 to 5% of the cells became rhodamine positive when L. monocytogenes was added at an MOI of 10. A simultaneous killing assay done with these cells showed that gp91$^{phox-/-}$ PMN were as capable of killing L. monocytogenes as cells from the wild-type NADPH oxidase-sufficient mice, regardless of the MOI used (Fig. 6A). Thus, in genetically deficient mice that were unable to produce ROS from birth, nonoxidative mechanisms were sufficient to kill L. monocytogenes.

As an alternate approach to block ROS activity in both BALB/c and B6 mice, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (4-hydroxy Tempo [Tempol]) was used to scavenge superoxide produced in response to L. monocytogenes. Tempol is a pleiotropic antioxidant with both superoxide dismutase- and catalase-like properties (40, 41). Gradient-enriched PMN were pretreated with Tempol for 90 min prior to the addition of L. monocytogenes, and DHR was added to the samples to report the generation of ROS. As shown in Fig. 6B, approximately 20 to 30% of either BALB/c or B6 PMN produced high levels of ROS within 30 min, and pretreatment with Tempol significantly reduced the percentage of ROS^{hi} cells detected. Tempol treatment also resulted in a 10 to 15% reduction in killing by PMN from both mouse strains (Fig. 6B). These data indicated that in mice genetically capable of producing ROS, scavenging oxidative intermediates (causing less hypochlorous acid to be available) reduced killing efficiency in PMN. However, these data were also consistent with the conclusion that oxidative killing mechanisms are not required to eliminate L. monocytogenes.

Since oxidative mechanisms were not required to kill L. monocytogenes, we hypothesized that inhibition of proteases such as elastase, cathepsin G, and proteinase 3 would result in at least partial loss of killing capacity. To test this, gradient-enriched
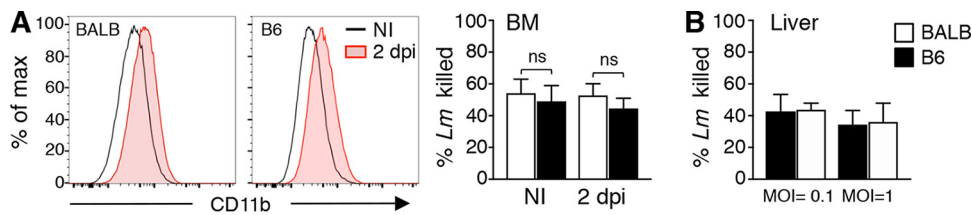
FIG 7 Neutrophils harvested from the bone marrow of infected BALB and B6 mice kill *Listeria* with similar efficiency. (A) CD11b surface expression on sorted PMN from uninfected mice (NI) or mice fed 3×10^8 CFU *L. monocytogenes* SD2000 (2 dpi) was determined. Representative flow cytometry histograms are shown. PMN were incubated with strain SD2001 for 50 min, and the percentage of kanamycin-resistant bacteria killed was determined. Bars indicate means $\pm$ SD for 6 technical replicates from one of three independent experiments. (B) BALB/c and B6 female mice were fed 1×10^9 CFU *L. monocytogenes*, and livers were harvested at 3.5 dpi following collagenase perfusion via the portal vein. The enriched nonparenchymal fraction was plated and exposed to strain SD2001 at the indicated MOI for 50 min, and the percentage of kanamycin-resistant bacteria killed was determined. Bars indicate means $\pm$ SD for 6 technical replicates from one of two independent experiments. ns, not significant.

PMN were incubated with diisopropylfluorophosphate (DFP), a nonspecific serine protease inhibitor. DFP was previously reported to inhibit protease activity without affecting production of ROS (42). Treatment with 5 mM DFP resulted in a significant reduction in PMN killing ability (Fig. 6C). However, treatment with 5 mM DFP also completely inhibited the ROS response in PMN exposed to *L. monocytogenes*. Lowering the concentration to 0.2 mM DFP eliminated the effect on ROS activity, but this concentration did not inhibit bacterial killing (Fig. 6C). Therefore, it was not possible to determine whether the loss of killing efficiency was due to a loss of serine protease activity or a reduction in ROS availability in wild-type mice. DFP treatment of PMN from gp91phox-deficient mice, however, did result in a significant loss of killing, suggesting that full protease activity is important for optimal killing in the absence of ROS (Fig. 6D).

PMN harvested from infected BALB/c and B6 mice kill *L. monocytogenes* with similar efficiency. Since murine PMN largely stay sequestered in bone marrow until receiving a chemotactic signal to egress into the circulation, the cells used in our *in vitro* assays were likely to be less activated than cells that are primed to infiltrate infected tissues. To assess the ability of activated cells to kill *L. monocytogenes*, we harvested bone marrow from BALB/c and B6 mice 2 days after foodborne infection with *L. monocytogenes*. At this time point, there are typically no CFU in the bone marrow; however, there are cells producing IFN-γ, and monocytes still in the marrow have undergone inflammatory changes (43). Likewise, PMN in the marrow of both BALB/c and B6 mice had uniformly upregulated CD11b expression (Fig. 7A). CD11b is a component of complement receptor 3, and it was previously shown that CD11b increased on the surface of human PMN after activation (44–46). However, no significant differences were observed for the ability of CD11bhi PMN to kill *L. monocytogenes* *in vitro* compared to cells from uninfected mice (Fig. 7A). Thus, unprimed cells from the marrow of uninfected mice behaved very similarly to primed cells from the marrow of infected mice.

To assess the ability of activated cells that had already emigrated from the bone marrow, we isolated PMN from infected livers. Collagenase-perfused livers were harvested from both BALB/c and B6 mice at 3.5 days after infection. PMN were gradient enriched, and then kanamycin-resistant *L. monocytogenes* was added. As shown in Fig. 7B, these cells killed 30 to 40% of the added *L. monocytogenes*. Together, these results suggest that there are no intrinsic differences in the ability of PMN from susceptible BALB/c or resistant B6 mice to clear *L. monocytogenes* infection.

DISCUSSION

To begin to address the possibility that susceptibility to listeriosis was determined by an intrinsic defect in PMN function in mice, we developed an *in vitro* killing assay using PMN enriched from murine bone marrow. We found that murine PMN killed opsonized *L. monocytogenes* efficiently even without being primed with agents such as

PMA or IFN-γ. Furthermore, murine PMN could be sorted and kept in culture for at least 24 h without affecting killing ability. We showed that there was no intrinsic difference in the ability of BALB/c and B6 PMN to kill *L. monocytogenes* directly *ex vivo*. However, it is possible that *in vivo*, there are substances produced in the BALB/c liver that inhibit the function of PMN and thus contribute to the delayed clearance of *L. monocytogenes*.

In agreement with previous studies using human PMN (33, 47), we showed that opsonization with naive serum significantly improved the ability of PMN to kill *L. monocytogenes*. *In vitro*, complement may be fixed on the surface of *L. monocytogenes* through the classical or alternative pathway (33, 48). *In vivo*, complement is rapidly activated during inflammation and may also be activated by either proteases or acute-phase proteins present during infection (49). The concentration of specific complement components in the liver during *L. monocytogenes* infection is unknown, but several studies have found increased expression of complement receptors on activated PMN (44–46). In humans, specific antibodies may also be involved in enhancing uptake and killing of *L. monocytogenes*. Using a rabbit model, Vahidy et al. demonstrated that *L. monocytogenes* opsonized with immune serum was internalized to a significantly greater extent than bacteria treated with nonimmune serum (50).

The ROS burst has long been thought of as a primary means by which PMN clear microbes, through oxidative damage to bacterial DNA, peroxidation of membrane lipids, and other mechanisms (37). ROS might also increase the K^+ concentration in vacuoles, raising the pH and releasing proteases from an anionic matrix to allow for optimal protease activity (51). Assembly of the multicomponent NADPH oxidase is triggered by the act of phagocytosis, resulting in targeted release of ROS (52, 53). However, in this study, only a high MOI induced robust intracellular ROS production in PMN and increased ROS activity did not result in more-efficient killing of *L. monocytogenes*. PMN from mice genetically unable to make ROS from birth had no defect in killing *L. monocytogenes*; therefore, nonoxidative killing mechanisms are sufficient. However, in animals capable of triggering an ROS burst, modulation of that response led to reduced bacterial killing. The loss of killing capacity observed after Tempol treatment was likely due to a disruption in the ratio of available oxygen intermediates. Since Tempol has catalase-like activity and also acts as a superoxide dismutase mimetic (40, 41), it presumably depletes the H_2O_2 that would normally be acted upon by myeloperoxidase to form hypochlorous acid, the oxidizing agent for DHR 123 (36).

It is not surprising that *L. monocytogenes* can be resistant to killing by oxidative mechanisms, as the bacteria are known to produce both catalase and superoxide dismutase as protection against host-derived ROS (54). Previously, stationary-phase *L. monocytogenes* bacteria were found to be more vulnerable to killing by human PMN than logarithmic-phase bacteria, a result that was attributed to lower catalase production (55). Furthermore, superoxide dismutase was found to be phosphorylated and thus less active in stationary-phase *L. monocytogenes* (56). However, in our study, we found no significant difference in the ability of murine PMN to kill either logarithmic-phase or stationary-phase *L. monocytogenes*, and both cultures produced similar bubbling reactions in the presence of hydrogen peroxide (M. G. Pitts, unpublished observation). The *L. monocytogenes* cytolysin listeriolysin O (LLO) was also shown to inhibit the activation of NADPH oxidase in macrophages (57); however, the toxin may be rapidly degraded in PMN (58).

PMN granules contain a variety of proteins and peptides that provide nonoxidative means of killing bacteria, including cathepsin G, elastase, proteinase 3, lactoferrin, and lysozyme (59). Alford et al. showed that purified cathepsin G alone could kill either *S. aureus* or *L. monocytogenes* (60). Since several of these compounds are serine proteases (60, 61), we expected to find at least a partial loss of killing efficiency after pretreatment with DFP, a serine protease inhibitor, but this occurred only in a gp91phox$^{-/-}$ background. DFP was previously used to show that serine proteases were important for human PMN to kill *Streptococcus pneumoniae* (62), but ROS activity was not monitored in that study, so we cannot make a direct comparison to our results. DFP was also used to inhibit proteolysis of CD43 on human PMN stimulated with opsonized zymosan; in

that study, the DFP did not inhibit ROS generation (42). This suggests that the nature of the phagocytosed particle may determine whether or not DFP impacts the ROS burst.

Circulating PMN are readily obtained from human peripheral blood; however, the cells are easily affected by *ex vivo* isolation and labeling techniques and are prone to rapid cell death (25, 63–65). In contrast, mature PMN harvested from the bone marrow have a longer half-life, can be maintained in culture for at least 24 h (26), and as we showed here, are not affected by manipulations such as cell sorting. A key advantage of using unprimed cells, rather than cells that have already egressed from the bone marrow, is the ability to study how specific inflammatory signals alter the functional properties of the PMN. An important challenge for future work will be to understand how signals in infected tissues such as the liver may affect the function of PMN during infection.

MATERIALS AND METHODS

Bacteria. Mouse-adapted (InlA^m-expressing) *L. monocytogenes* EGDe derivatives SD2000, SD2001 (kanamycin resistant), and SD2710 (constitutive GFP) were used in this study (43, 66). A freshly streaked colony was inoculated into brain heart infusion (BHI) broth (Difco) and grown with shaking at 37°C overnight (16 h). For *in vitro* assays, *L. monocytogenes* cells were washed once with Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS; Gibco) and then diluted in the same buffer. For serum pretreatment, autologous mouse serum was added (final concentration, 10%) for 30 min. Pretreatment with serum alone in the absence of PMN had no effect on *L. monocytogenes* growth (data not shown). Heat-inactivated serum was incubated at 55°C for 30 min.

Mice. BALBcBy/J (BALB/c; stock number 001026), C57BL/6J (B6; stock number 000664), and B6.129S-*Cybb^{tm1Din}*/J (gp91^phox−/−; stock number 002365) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a specific-pathogen-free facility. Bone marrow was harvested from both male and female animals 8 to 16 weeks old; no significant differences were noted in PMN recovery per gram of weight between male and female mice. Blood was collected from the aorta immediately after euthanasia and transferred to a serum separator tube (BD), followed by centrifugation for 2 min at 20,000 × *g*. Serum was stored at 4°C for use the same day or at −80°C for later use. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Liver nonparenchymal cell preparation. Mice were fed bread contaminated with *L. monocytogenes* SD2000 as described previously (67, 68); control mice were fed phosphate-buffered saline (PBS)-saturated bread pieces. Livers were perfused via the hepatic portal vein with 1 ml of collagenase type IV solution (250 U/ml in HBSS; Worthington), removing blood present in the tissue. The perfused tissue was cut into small pieces, transferred to a 50-ml tube containing DNase (10 U/ml; Worthington) and collagenase type IV (100 U/ml) in 10 ml of RP-5 medium (RPMI 1640 [Life Technologies number 21870], 5% fetal bovine serum [FBS; Gemini], 2.5 mM L-glutamine, 10 mM HEPES, and 0.1 mM β2-mercaptoethanol [Sigma-Aldrich]), and incubated for 35 min with shaking at 37°C. The digested tissue was gently pushed through a mesh screen (number 80 mesh) to create a single-cell suspension. At this point, 1/10 of the volume was removed for determination of CFU burdens. Nonparenchymal cells were enriched by allowing 2 min of settling time followed by centrifugation for 1 min at 50 × *g* at 4°C. Supernatant was centrifuged at 300 × *g* at 4°C for 10 min, the pellet was suspended in 30 ml of RPMI 1640, and the prior two steps were repeated. Finally, the pellet was suspended in 1.6 ml cold RPMI 1640 followed by addition of 2.4 ml cold 40% Histodenz (Sigma) in PBS. The suspension was layered under 2 ml of cold RPMI 1640 in 15-ml polyethylene terephthalate (PET) tubes (Corning) that had been precoated with FBS. Samples were centrifuged for 20 min at 4°C at 1,500 × *g* with no brake. The interface was collected and passed through a filter to remove clumps.

Bone marrow harvest. Marrow was flushed from femurs and tibias with medium containing 2 μM EDTA (Gibco) and 25 μg/ml gentamicin (Gibco). Cells (two bones per tube) were passed through a sterile mesh filter into a 15-ml PET tube and pelleted by centrifugation at 400 × *g*. Erythrocytes were lysed by exposure to 0.2% NaCl for 20 s followed by addition of an equal volume of 1.6% NaCl.

Density gradient enrichment of PMN. Following erythrocyte lysis, PMN were enriched as described previously (27). The bottom layer of cells was recovered and suspended in RP-10 (same as RP-5 but with 10% FBS) or flow cytometry buffer (Ca^{2+}/Mg^{2+}-free HBSS, 25 mM HEPES, 5 mM EDTA, 1% FBS).

Flow cytometry. Cells were stained using fluorescently conjugated antibodies specific for the following molecules: Ly6C (HK1.4) and Ly6G (1A-8), purchased from Biolegend; CD11b (M1/70), CD3 (500A2), B220 (RA3-6B2), CD64 (X54-5/7.1), and CD19 (1D3), purchased from eBioscience. Neutrophils were defined as Ly6G^hi Ly6C^{+/−}, monocytes as Ly6G^− Ly6C^hi, and B cells as Ly6G^− Ly6C^− CD19^+. The sort gating strategy incorporated live-cell and singlet gates prior to gating on individual markers; sort purities for PMN ranged from 92 to 99%. Cells were sorted into flow cytometry buffer containing 50% FBS and 25 μg/ml gentamicin and then washed twice with RP-10 medium. Fluorescence was measured using an LSR II flow cytometer (BD Biosciences) or an iCyt Synergy sorter, and analysis was performed using FlowJo v.10 (Tree Star).

In vitro killing assay. Neutrophils (10^5/well) were plated in 96-well flat-bottom tissue culture-treated plates in 100 μl in RP-10 and incubated at 37°C in 7% CO_2 for 1 h to allow a period of recovery. *L. monocytogenes* was added at the indicated MOI in a volume of 20 to 40 μl to 3 to 6 wells per sample

group. In each experiment, a group of wells contained bacteria only (no PMN) to assess growth/survival. The plate was centrifuged at $300 \times g$ for 5 min and then incubated at 37°C in 7% CO_2. At each time point, the content of each well was transferred to individual microcentrifuge tubes and wells were vigorously washed 3 times with PBS and examined microscopically to ensure that all cells were removed. Serial dilutions of each well were prepared in PBS and plated on BHI agar (Difco). The percentage of *L. monocytogenes* killed was calculated by dividing the number of CFU from wells with PMN by the mean number of CFU from wells without PMN.

Gentamicin protection assay. Gradient-enriched PMN were plated and exposed to serum-opsonized *L. monocytogenes* as described above. Gentamicin was added at a final concentration of 10 µg/ml 10 min later. At indicated time points, the cells were washed once with RP-10, lysed by addition of sterile water, and plated on BHI agar.

Chemotaxis assay. An under-agarose assay was used as described previously by Heit and Kubes (69), with the exception of the following modifications. Ultrapure agarose (1%; Invitrogen) was dissolved in Ca^{2+}/Mg^{2+}-free HBSS and diluted with phenol red-free RPMI 1640 containing a final concentration of 2.5% FBS. FBS-coated 60-mm petri dishes were filled with 4.5 ml of the agarose mixture. Three-millimeter holes were cored 2 mm apart using a sterilized template made from a silicone sheet and a hollow punch tool. Either 1 µM formylated synthetic peptide (fMIVTLF plus fMIGWII; Bio-Synthesis, Inc.) or 100 nM leukotriene B4 (Cayman Chemical) diluted in Ca^{2+}/Mg^{2+}-free HBSS was applied to the center well and allowed to diffuse into the gel prior to addition of gradient-enriched PMN (2×10^5/well) to the outer wells. Dishes were incubated at 37°C in 7% CO_2 for 3 h. Images were acquired in differential interference contrast (DIC) mode with a Nikon A1R confocal microscope, using a 10× air objective with a numerical aperture of 0.45 and the transmitted light detector. Image seize was 4,096 by 4,096 pixels with a resolution of 0.31 µm/pixel. The distance traveled was determined using the manual measurement tool in the NIS-Elements software program (Nikon).

Immunofluorescence microscopy. Sorted PMN were plated in RP-10 and rested for 1 h, and then cytochalasin D (Sigma) (20 µM in dimethyl sulfoxide [DMSO]) was added and cells were incubated for 45 min at 37°C in 7% CO_2. *L. monocytogenes* SD2710 was added to the wells, and the plate was centrifuged for 5 min at $300 \times g$, followed by incubation for 5 min at 37°C in 7% CO_2. Difco Listeria O Antiserum Poly (BD Biosciences) and goat anti-rabbit IgG-Texas Red (Thermo Fisher) were used to perform differential "in/out" staining as described previously (43). Cells were visualized using a Zeiss Axio Imager.Z1 with a 100×/1.4 NA PlanApo oil immersion objective and analyzed with AxioVision software. Slides were blinded and examined by two different investigators; average values are reported.

Reactive oxygen species. Gradient-enriched PMN were plated in RP-10, rested for 1 h, and then centrifuged at $300 \times g$ and suspended in Ca^{2+}/Mg^{2+}-free HBSS. Dihydrorhodamine 123 (DHR; Cayman Chemical number 85100) suspended in DMSO and diluted in HBSS was added to wells (final concentration, 2.5 µg/ml) 15 min prior to addition of either *L. monocytogenes* or 20 nM PMA diluted in HBSS (Sigma catalog number P1585). Plates were centrifuged at $300 \times g$ for 5 min and then incubated at 37°C in 7% CO_2. To scavenge reactive oxygen intermediates, cells were incubated for 90 min with 50 mM 4-hydroxy Tempo dissolved in HBSS (Tempol, Sigma catalog number 176141). *L. monocytogenes* was added at the indicated MOI, and the mixture was further incubated for 30 min. DHR was added during the final 15 min of incubation.

Serine protease inhibition. Cells were treated with diisopropylfluorphosphate (DFP; Sigma catalog number D0879), suspended in isopropanol, and diluted in HBSS for 30 min at 37°C in 7% CO_2. DHR was added to the wells prior to addition of *L. monocytogenes*. For flow cytometry analysis, cells were washed, stained with fluorescently tagged anti-Ly6G antibody, and fixed in 10% formalin after incubation with bacteria.

Statistical analysis. Statistical analysis was performed using Prism 6 for Macintosh. Unless otherwise indicated, unpaired Student's *t* tests were performed. *P* values of <0.05 were considered significant and are indicated in the figures by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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