Innate immune-induced depletion of bone marrow neutrophils aggravates systemic bacterial infections

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Neutrophils are the most abundant leukocytes in circulation and provide a primary innate immune defense function against bacterial pathogens before development of a specific immune response. These specialized phagocytes are short lived (12–24 hours) and continuously replenished from bone marrow. We found that if the host is overwhelmed by a high inoculum of *Listeria monocytogenes,* neutrophils are depleted despite high granulocyte-colony stimulating factor induction. In contrast to a low-dose innocuous *L. monocytogenes* infection, high-dose Listeria challenge blocks neutrophil recruitment to infectious abscesses and bacterial proliferation is not controlled, resulting in lethal outcomes. Administering synthetic TLR2-ligand or heat-killed bacteria during the innocuous *L. monocytogenes* infection reproduced these effects, once again leading to overwhelming bacterial propagation. The same stimuli also severely aggravated *Salmonella typhimurium*, *Staphylococcus aureus,* and *Streptococcus pyogenes* systemic infection. These data implicate systemic innate immune stimulation as a mechanism of bone marrow neutrophil exhaustion which negatively influences the outcome of bacterial infections.

Listeria monocytogenes | neutropenia | TLR2 | sepsis | exhaustion

Neutrophils provide an important early defense mechanism against infection. Under normal circumstances, only a small fraction of the neutrophil pool are found in circulation (<2% of 65 million in the mouse), whereas most are stored in the bone marrow (BM) (1, 2). In response to infection, BM neutrophils are released and control invading pathogens in the periphery via phagocytosis, oxidative agents, enzymatic digestion, and formation of extracellular traps. Neutrophils die in the process of bacterial killing (3), and granulocyte-colony stimulating factor (G-CSF) is up-regulated to induce granulopoiesis and to replenish the BM reservoir (4).

Interruption of the neutrophil supply is detrimental to the control of bacterial infections (5, 6). Absence of circulating neutrophils or, conversely, abnormal elevation of their numbers, can each be indicators of bacterial sepsis (7). How these apparently contradictory clinical signs correlate to underlying events within the BM neutrophil reservoir remains poorly understood. The mechanisms responsible for the high mortality of sepsis are also unclear, although generally systemic over-activation of immune response mechanisms coupled with a failure to fully eradicate bacteria, are blamed (8, 9).

In the present work, we used varying doses of *Listeria monocytogenes* (L.m.) (10) to initiate an innocuous versus a lethal infection, and studied the relationship between the inoculum size, neutrophil kinetics, and host survival. Only a lethal dose of L.m. devastated the BM neutrophil supply through excessive demand and accelerated cell death. Exploration of these results uncover a connection between systemic innate stimulation as exemplified by Toll-like receptor 2 (TLR2) activation, BM neutrophil exhaustion, and mortality, further corroborated through pharmacologic and genetic manipulations and systemic challenge with different bacterial species. Taken together, our data connect innate immune activation to BM neutrophil exhaustion, which we identify as a critical risk factor for fulminant bacterial infections and fatal outcomes.

Results

Bone Marrow Neutrophils Are Not Depleted During Low-Dose L.m. Infection. The neutrophil response in inbred C57BL/6 wild-type (WT) mice to an innocuous, low-dose infection with 10^3 colony forming units (cfu) L.m. i.v. (≈1/10 of the median lethal dose, LD_{50}) was analyzed. Visualization of the time course of this infection by bacterial counts in spleens and livers showed a rise of bacterial loads until day 3 before these fell below the detection limit at day 9 (Fig. 1*A*). Of the three hematologic parameters measured, neither blood nor BM neutrophils nor BM macrophages (11, 12) showed significant alterations during the first 5 days of the infection. Only at days 7 and 9 were BM neutrophils found to be elevated (Fig. 1*B*). All low-dose–infected animals survived for at least 30 days (Fig. 1*C*). Low-dose infection stimulated granulopoiesis, and G-CSF levels rose to a maximum of ≈10 ng/ml between days 1 and 2, before returning to undetectable levels by day 7 (Fig. 1*D*).

Depletion of BM Neutrophils During Lethal L.m. Infection. To contrast these observations to a lethal high-dose infection with the same bacterium, mice were challenged with a dose of 10^5 cfu L.m. i.v. (≈10 times LD_{50}). This high-dose infection caused an accelerated decrease in BM neutrophils, until by day 3 less than 10% of the baseline number remained (Fig. 1*E*). Neutrophil depletion was not averted by the strong and immediate induction of G-CSF (Fig. 1*D*). BM macrophage numbers showed little or no change, suggesting that depletion was granulocyte-specific (Fig. 1*E*). Splenic and hepatic bacterial counts (Fig. 1*F*) showed a significant negative correlation with mean BM neutrophil numbers in both low- and high-dose infection [supporting information (SI) Figs. S1 *A* and *B*]. Over the course of 3 days, the bacterial numbers in spleen, liver, and BM kept rising steadily, and animals developed bacteremia (Fig. 1*F*). By day 3, nearly half the mice had developed end-stage disease, and the remaining animals had to be taken out of the experiment by day 5 because of terminal illness (Fig. 1*C*).

BM Exhaustion Coincides with Halting Neutrophil Infiltration into Bacterial Lesions. Histological analysis of WT livers and spleens revealed distinct immune cell infiltration kinetics in response to low- or high-dose L.m. infection. During low-dose infection, strong hepatic (Fig. 2*A*) and splenic (Fig. 2*B*) neutrophil infil-

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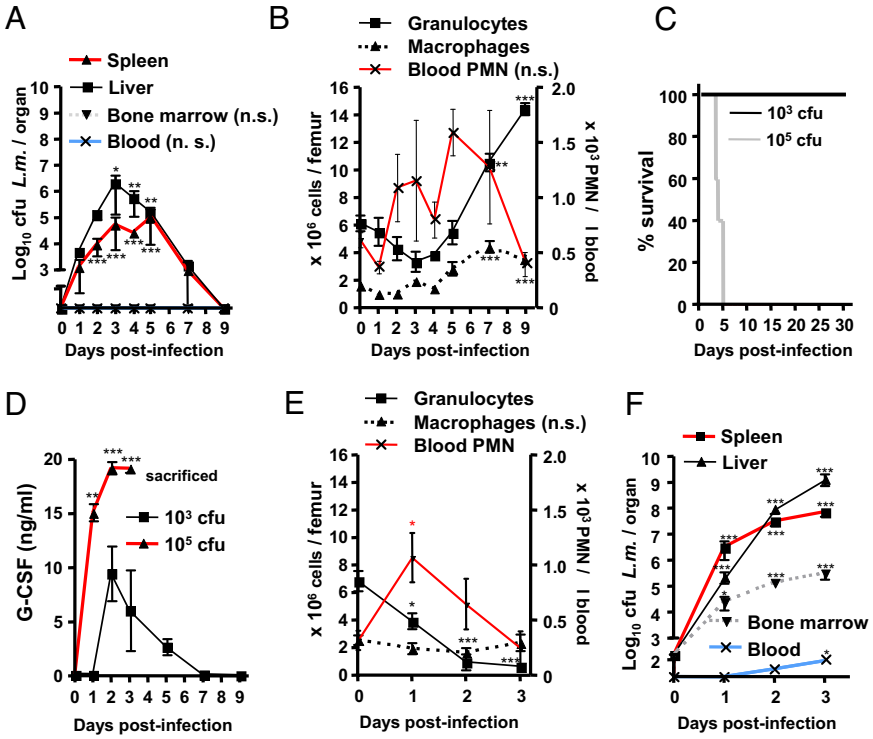

Fig. 1. Transient versus terminal depletion of BM neutrophils by low- or high-dose *Listeria monocytogenes* (L.m.) infection. (*A*) L.m. counts in spleen, liver, BM (cfu/femur), and blood (cfu/μl) at the respective timepoints ($n = 4$ per timepoint, mean ± SEM, one of two experiments with similar outcomes). (*B*) Ly6G$^+$CD11b$^+$ (neutrophils) and CD11b$^+$Ly6G$^-$ (macrophages) cells in BM and Ly6G$^+$CD11b$^+$ (neutrophils) in blood at the respective days after low-dose L.m. infection (10^3 cfu) ($n = 3$–4 per time point, mean ± SEM, one of two experiments with similar outcomes) (*C*) Survival of WT mice after infection with low-dose (10^3 cfu) or high-dose (10^5 cfu) L.m. i.v. ($n = 5$–10/group) (*D*) Serum concentration of G-CSF after infection low-dose (10^3 cfu) or high-dose (10^5 cfu) L.m. i.v. ($n = 3$–4 per time point, mean ± SEM) (*E*) Ly6G$^+$CD11b$^+$ (neutrophils) and CD11b$^+$Ly6G$^-$ (macrophages) cells in BM and Ly6G$^+$CD11b$^+$ (neutrophils) in blood at the respective days after infection with 10^5 cfu L.m. ($n = 3$–4 per time point, mean ± SEM, one of two experiments with similar outcomes) (*F*) Bacterial counts in spleen, liver, BM (cfu/femur), and blood (cfu/μl) at the respective timepoints ($n = 4$ per timepoint, mean ± SEM, one of two experiments with similar outcome)

tration occurred between days 3 and 5 but reverted to near-basal levels by day 9, when bacteria were fully cleared from the organs (Fig. 1*A*). Over the course of both low- and high-dose infections, F4/80-staining intensity of splenic macrophages decreased slightly, consistent with cellular activation (13), but macrophage numbers and distribution patterns did not change noticeably (Fig. S2 *B* and *D*). In the liver, F4/80$^+$ Kupffer cells and macrophages started to infiltrate the abscesses from day 3–4 onward (Fig. S2 *A* and *C*). Levels of L.m. remained under the immunohistochemically detectable limit for the entire duration of the low-dose infection in both liver and spleen (Fig. S2 *A* and *B*). In contrast, in high-dose L.m. infection, dense neutrophil infiltrates were observed in liver and spleen as early as day 1 (Fig. 2 *C* and *D*), 2 days earlier than during low-dose infection (Fig. 2 *A* and *B*). However, by day 3, neutrophils had all but disappeared from the organs, coinciding with the exhaustion of BM neutrophils (Fig. 1*E*), peak L.m. organ counts, histologically detectable bacteria (Fig. S2 *C* and *D*), and bacteremia (Fig. 1*F*). Hepatocytes stained especially strongly for dense clusters of bacteria by day 3 (Fig. S2*C*), when terminal illness set in (Fig. 1*C*), whereas in the spleen a more diffuse L.m. distribution pattern was observed (Fig. S2*D*).

Lethal Bacterial Infection Causes Death of BM Neutrophils. To determine the degree to which L.m. infection caused leukocyte activation, expression levels of early activation marker CD11b (14) on BM and blood neutrophils were measured. Both high- and low-dose inocula caused neutrophil activation in BM (Fig. 3 *A* and *B*). However, whereas activation levels during low-dose infection peaked between days 3 and 5 and reverted to baseline levels (Fig. 3*A*), neutrophil activation in high-dose–infected mice kept increasing (Fig. 3*B*) until end-stage disease set in (Fig. 1*C*).

As activated neutrophils limit their potential for immunopathology by eventually undergoing apoptosis (15), Annexin-V stainings were performed. In the blood, virtually no dead neutrophils were found at any time (not shown). Strikingly, however, although the levels of dead BM neutrophil remained <10% in the low-dose–infected animals (Fig. 3*C*), high-dose infection resulted in 50% and 70% of dead BM neutrophils on days 2 and 3, respectively (Fig. 3*D*).

Innate Immune Activation Triggers BM Neutrophil Exhaustion. To determine whether the poor outcome of high-dose infection was caused solely by the higher replication potential of the larger inoculum, or whether it could also be caused by nonproliferative bacterial constituents, heat-killed L.m. were administrated a day after low-dose infection. At day 3, bacterial counts in liver and spleen were one to two orders of magnitude higher in animals that received heat-killed bacteria (Fig. S3*A*), providing a first indication that non-proliferative bacterial constituents might play a role in granulocyte depletion.

Lipopeptides and lipoproteins of Gram-positive bacteria such as L.m. can be recognized by the pattern recognition receptor TLR2 to activate the host innate immune response (16). Therefore, we administered 100 μg of the synthetic bacterial lipopeptide and TLR2 ligand Pam2Cys intravenously into WT or *tlr2*$^{-/-}$ mice to test whether TLR2 (17) would provide a route by which inert bacterial constituents could induce granulocyte depletion. Although they remained free of symptoms, strikingly, Pam2Cys-treated WT mice showed a >80% BM neutrophil reduction

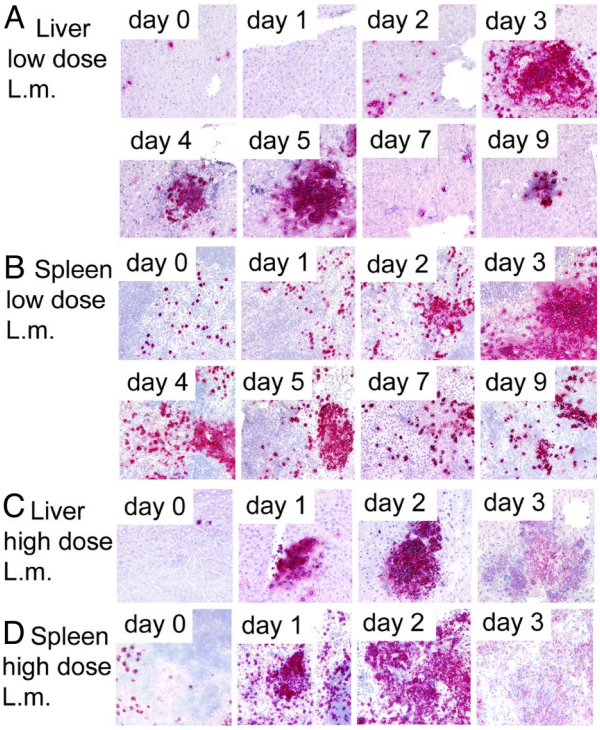

Fig. 2. Histological time course of neutrophils in livers and spleens during infection with low- or high-dose L.m. Livers and spleens taken at the indicated time points were stained immunohistochemically for presence of GR-1$^+$ cells, in (*A*) and (*B*) during infection with 10^3 cfu L.m. and in (*C*) and (*D*) with 10^5 cfu L.m.

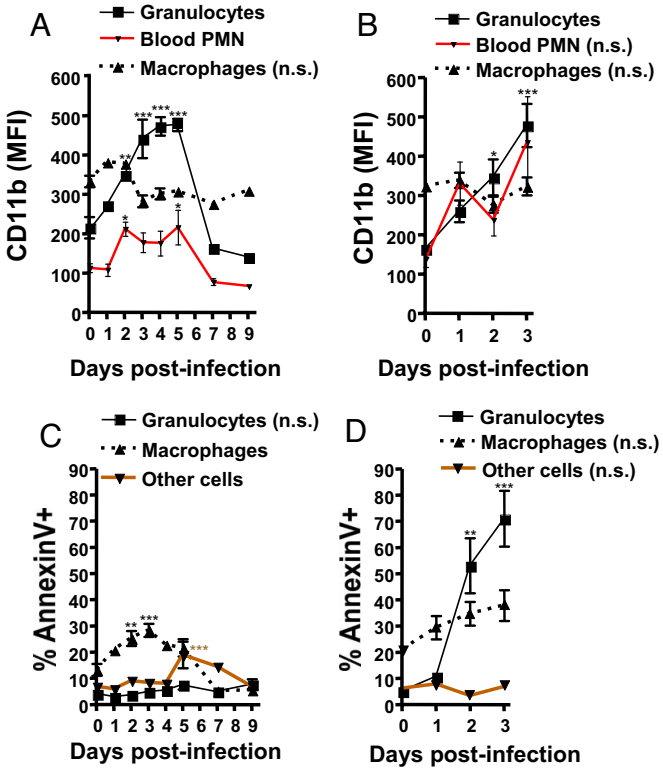

Fig. 3. Activation and death of neutrophils during infection with low- and high-dose L.m. (*A* and *B*) Mean fluorescence intensity of CD11b on the surface of Ly6G$^+$CD11b$^+$ (neutrophils) and CD11b$^+$Ly6G$^-$ (macrophages) cells in BM and blood after infection with 10^3 cfu (*A*) or 10^5 cfu L.m. (*B*) (n = 3–4, mean ± SEM, one of two experiments with similar outcomes) (*C* and *D*) Percentage of Annexin-V$^+$ neutrophils, macrophages, and Ly6G$^-$CD11b$^-$ cells (other cells) in BM after infection with 10^3 cfu (*C*) or 10^5 cfu (*D*) L.m. (n = 3–4, mean ± SEM, one of two experiments with similar outcomes)

compared with *tlr2*$^{-/-}$ mice and WT controls (Fig. 4 *A* and *B*). Moreover, and in contrast to other studies correlating BM neutrophils with viral and bacterial infection (18), TLR2-mediated neutrophil depletion was independent of interferon (IFN) type I (Fig. S3*B*). Up to 40% of BM neutrophils underwent apoptosis in WT mice but not in *tlr2*$^{-/-}$ controls (Fig. 4*C*). To investigate whether, in addition to apoptosis, accelerated emigration contributed to the Pam2Cys-induced BM neutrophil reduction, neutrophil numbers in the blood and major filtering organs (liver and spleen) were determined. Although no significant changes in either viable or apoptotic granulocytes were observed in liver or blood (Fig. S3 *C* and *D*) 6-fold more apoptotic neutrophils accumulated in spleens (19) of Pam2Cys-treated WT animals than in those of PBS-treated controls (Fig. 4*D*). In addition, only BM neutrophils of WT mice showed up-regulation of early activation marker CD11b (Fig. 4*E*) and demonstrated respiratory burst activity (Fig. 4*F*) in response to Pam2Cys. Interestingly, *tlr2*$^{-/-}$ mice showed heightened neutrophil activation even in the naïve state, which has not yet been analyzed further. To determine whether this phenomenon could also be induced by other TLRs, we administered the prototypic TLR4 ligand LPS to C57BL/6 mice and found a similar rate of dead neutrophils as well as numerical reduction in the bone marrow (not shown).

Systemic TLR2 Activation Converts Low-Dose Bacterial Infection into Lethal Sepsis. To investigate the influence of specific TLR2 engagement on an innocuous infection, WT mice or *tlr2*$^{-/-}$ controls were infected with low-dose L.m. and treated with Pam2Cys i.v. (Fig. 5*A*). Although *tlr2*$^{-/-}$ mice and WT PBS controls remained free of disease, WT animals treated with Pam2Cys became terminally ill between days 3 and 5 (Fig. 5B), reminiscent of non-Pam2Cys–treated but high-dose–infected animals (Fig. 1*C*). At day 3, organs of Pam2Cys treated WT animals showed one to two orders of magnitude higher bacterial counts than their PBS controls, whereas *tlr2*$^{-/-}$ animals were resistant to the aggravating effects of Pam2Cys (Fig. 5*C*). High-dose–infected, Pam2Cys-treated WT mice had fewer neutrophils left in their bone marrow and blood (not shown) at day 3 compared with PBS and *tlr2*$^{-/-}$ controls (Fig. 5*D*). Treatment with granulocyte-depleting α-GR1 antibody led to the same constellation of findings as with Pam2Cys treatment (20, 21) (Fig. 5 *C* and *D*). Histologically, livers of *tlr2*$^{-/-}$ mice and WT PBS controls revealed densely packed infiltrating neutrophils at day 3 of infection, but only few bacteria. In contrast, Pam2Cys-treated WT animals showed multiple L.m. foci that were nearly devoid of infiltrating neutrophils (Fig. 5*E*).

Finally, we determined whether systemic TLR2 activation and BM granulocyte depletion would have similar effects in other bacterial infections. Pam2Cys administration significantly increased bacterial counts by one to two orders of magnitude in multiple organs after systemic infection with the important Gram-negative pathogen *Salmonella typhimurium* (liver, spleen) as well as the leading Gram-positive pathogens *Streptococcus pyogenes* (blood, spleen, liver) and *Staphylococcus aureus* (blood, spleen, brain) (Fig. 5 *F*–*H*). Thus TLR2 activation was found capable of aggravating the severity of systemic infection by multiple pathogens.

Discussion

It is generally believed that immune over-activation is a major contributor to the lethality of sepsis (22, 23). High cytokine concentrations (24) as seen in the systemic inflammatory reac-

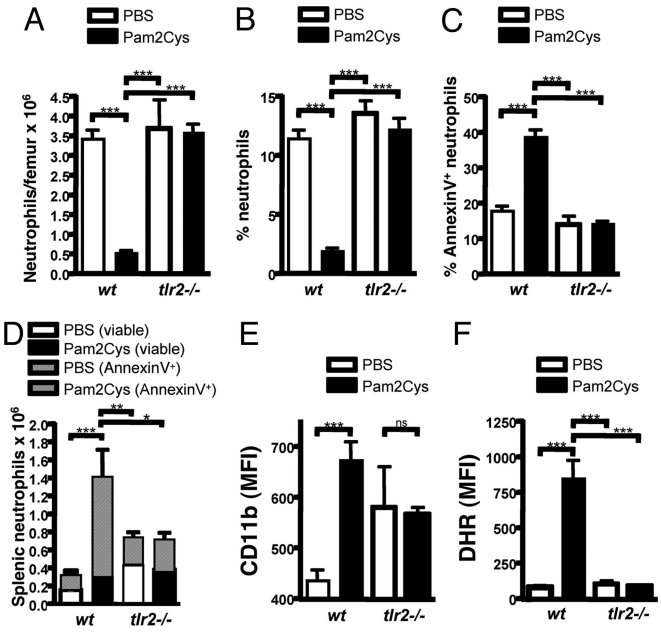

Fig. 4. BM neutrophil depletion 1 day after systemic TLR2 stimulation. (*A* and *B*) BM neutrophils (Ly6G$^+$CD11b$^+$) numbers (*A*) and percentages (*B*) measured by FACS 24 hours after administration of 100 μg Pam2Cys i.v. (mean ± SEM, n = 3–5 animals/group, one of two experiments with similar outcomes). (*C*) Percentage of dead (Annexin-V$^+$) neutrophils in BM 24 hours after 100 μg Pam2Cys i.v. (mean ± SEM, n = 3–5 animals/group, one of two experiments with similar outcomes). (*D*) Number of neutrophils in spleens 24 hours after administration of 100 μg Pam2Cys. Lower bars show viable (7AAD$^-$Annexin-V$^-$) neutrophils; upper bars show Annexin-V$^+$7AAD$^{+/-}$ neutrophils (mean ± SEM, n = 3–5 animals/group, one of two experiments with similar outcomes). (*E*) Mean fluorescence intensity of CD11b surface staining on BM neutrophils 24 hours after administration of 100 μg Pam2Cys i.v. (mean ± SEM, n = 3–5 animals/group, one of two experiments with similar outcomes). (*F*) Mean fluorescence intensity of DHR123 staining in BM neutrophils 24 hours after administration of 100 μg Pam2Cys i.v. (mean ± SEM, n = 3–5 animals/group, one of two experiments with similar outcomes).

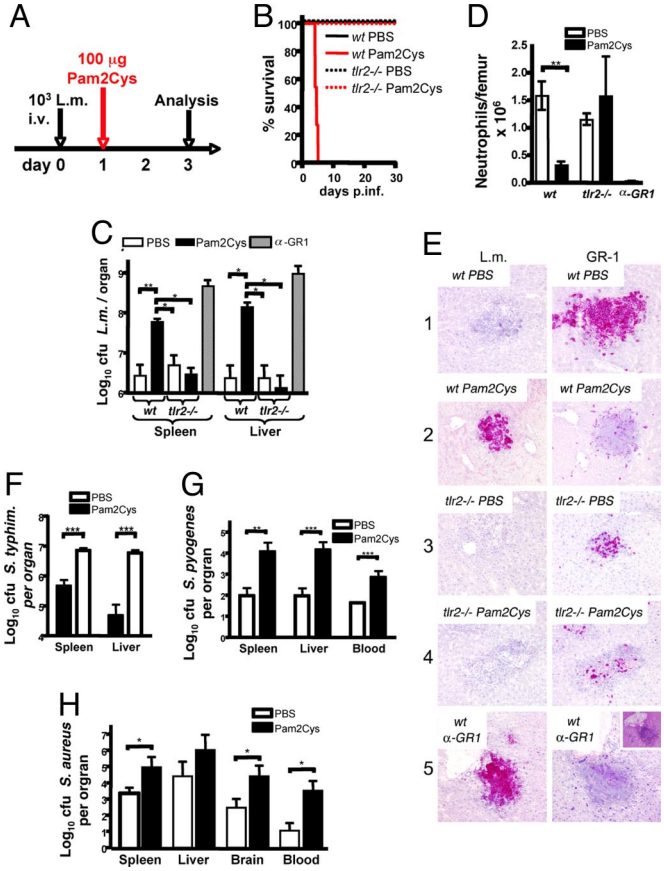

Fig. 5. Systemic TLR2 ligation leads to impaired neutrophil infiltration, uncontrolled bacterial propagation, and host death. (*A*) Experimental protocol. (*B*) Survival curve of *tlr2*$^{-/-}$ or WT mice infected with 10^3 cfu L.m. with or without administration of 100 μg Pam2Cys 24 hours after infection. (n = 5–11/group, two experiments) (*C*) Bacterial counts at day 3 of infection with 10^3 cfu L.m. with or without administration of 100 μg Pam2Cys 24 hours after infection. Grey bars show WT controls that had received 100 μg α-GR1 antibody (clone NimpR14) at 24 hours after infection (mean ± SEM, n = 3 per group, one of two experiments shown with similar outcomes). (*D*) BM neutrophils at day 3 of the L.m. infection (mean ± SEM, n = 3, one of two experiments with similar outcomes). (*E*) Immunohistochemistry of livers at day 3 of Listeria infection stained with anti-L.m. serum (left row) or α-GR1 (neutrophils, right row). Conditions shown are WT PBS (1), WT Pam2Cys (2), *tlr2*$^{-/-}$ PBS (3), *tlr2*$^{-/-}$ Pam2Cys (4), WT α-GR1 with hematoxylin and eosin–stained inlet (5) to confirm specific staining and absence of neutrophils. (*F*) Bacterial counts at day 3 of infection with 4×10^4 cfu *Salmonella typhimurium* with or without administration of 100 μg Pam2Cys 24 hours after infection (mean ± SEM, n = 5/group). (*G*) Bacterial counts at day 3 of infection with 5×10^6 cfu *Streptococcus pyogenes* with or without administration of 100 μg Pam2Cys 24 hours after infection (mean ± SEM, n = 10/group, two experiments pooled). (*H*) Bacterial counts at day 3 of infection with 5×10^6 cfu *Staphylococcus aureus* with or without administration of 100 μg Pam2Cys 24 hours after infection (mean ± SEM, n = 10 per group, two experiments pooled).

tion syndrome SIRS (considered a cytokine storm subtype) (7) have been associated with high mortality during sustained bacteremia. Furthermore, elevated levels of specific cytokines, e.g., type I IFN, have been linked to neutropenia (18), which by itself is an important predictor of poor outcomes of systemic bacterial infections (25). Nonetheless, specific underlying mechanisms of lethal outcomes of bacterial infections remain poorly understood (8), as perhaps best illustrated by the fact that therapies seeking to down-modulate key inflammatory mediators (26) or to boost granulopoiesis (27) during sepsis have generally not proved as successful as hoped.

Our data illustrate that the proliferative potential of the bacterial inoculum (i.e., the infectious dose) is merely one factor in lethal sepsis. The innate immune status of the host is another. Excessive innate activation through TLR2 can have a substantially detrimental effect on granulocyte-mediated antibacterial resistance. The systemic presence of bacterial compounds deriving from the infection, but also TLR2-triggering factors unrelated to the infection, were found to exhaust the BM neutrophil reservoir through a combination of increased demand and increased cell death. Infection-independent innate immune triggering through TLR2 aggravated a normally innocuous infection with L.m., *S. aureus*, *S. pyogenes*, or *S. typhimurium* to stages that are, under typical experimental conditions, only reached using lethal challenge dosages of these bacteria. Although out of the scope of the research presented here, we suggest that this mechanism of aggravating otherwise innocuous infections might also play a role in the high rates of secondary sepsis in patients whose innate immune system is stimulated during trauma, operative procedures, or burn injury.

In an earlier report, we showed how virus infection depleted BM neutrophils in a IFN type I–dependent manner, and worsened the outcome of a bacterial superinfection (18). In contrast to that finding, we describe here a type I IFN–independent mechanism of BM neutrophil depletion, as IFN I receptor–deficient mice showed no resistance to the BM depletion in response to Pam2Cys-treatment.

Recombinant G-CSF has been used to treat sepsis, under the premise that autologous production of G-CSF would be insuf-

ficient (27, 28). Although such treatment might be beneficial if normal production were impaired, G-CSF levels did not seem to be a limiting factor in WT mice (Fig. 1*D*). In fact, we found that G-CSF secretion correlated inversely with the size of the BM neutrophil reserve. Apparently, even strongly up-regulated G-CSF levels remained ineffective in generating the neutrophil quantity required for survival in the face of an overwhelming bacterial infection. This could explain the failure of therapeutic G-CSF or GM-CSF during sepsis (27). Still, given the innocuous effects of general G-CSF treatment, pre-emptive induction of granulopoiesis (29), for example, before elective surgery, might be beneficial by providing a narrow therapeutic window of elevated resistance.

Taken together, without contradicting other findings, our results shed light on the poorly understood mechanisms behind lethal sepsis, demonstrating that TLR-mediated innate immune stimulation worsens the outcome of bacterial infection by exhausting the BM neutrophil supply.

Materials and Methods

Mice. Specific pathogen free (SPF) laboratory mice were obtained from the institute of Labortierkunde of the veterinary facility of the University of Zurich. Experiments were performed according to Swiss veterinary law and institutional guidelines. C57BL/6 (WT) mice, *tlr2*$^{-/-}$ and *ifnar*$^{-/-}$ mice on C57BL/6 genetic background were used. For staphylococcal and streptococcal infections, WT C57BL/6 mice were obtained from Charles River Laboratories, and experiments performed under protocols of the Committee on the Use and Care of Animals at UCSD using accepted veterinary standards.

Infections. *Listeria monocytogenes* (L.m.) strain 10403S was grown overnight in brain–heart infusion broth or thawed from frozen aliquots, washed two times in phosphate-buffered saline (PBS), and injected i.v. in 200 μl into the tail vein. A 10^3-cfu quantity of L.m. i.v. ($^1/_{10}$ LD_{50}) was used as low-dose infection, and 10^5 cfu L.m. i.v. as high-dose infection. Heat-killed L.m. were 10^8 cfu L.m. heated at 70 °C for 1 hour and administrated i.v. *Salmonella typhimurium* (S.t.) was grown overnight in LB medium and washed in PBS, and 4×10^4 cfu injected i.v. in 200 μl. *Staphylococcus aureus* Newman ATCC 25904 and *Streptococcus pyogenes* M1T1 strain 5448 were grown to logarithmic phase and 5×10^6 cfu were injected i.v., respectively. Administered lipopeptide was Pam2Cys, a *Mycoplasma fermentans*–derived lipopeptide consisting of palmitoyl side chains acid and a cysteine, S-(2,3-bis(palmitoyloxy)propyl)cysteine (30).

Bacterial Counts. Bacterial counts were determined in homogenized halves of spleen, the left lobe of the liver, the brain, 100 μl of flushed BM (3 ml PBS per femur), or 50 μl of PBL in PBS with serial dilutions plated on brain–heart infusion (L.m.), blood (S.t.) agar, or Todd-Hewitt agar plates (*S. aureus* and *S. pyogenes*).

BM Aspirates and Culture. BM cells were flushed with PBS from the femur and were stained for fluorescence-activated cell sorting (FACS) analysis or cultured in RPMI with 10% fetal calf serum.

FACS and Antibodies. α-Ly6G, GR1 (Hybridoma RB6 8C5), CD11b, Annexin-V, and 7-Amino-Actinomycin-D (7-AAD) were obtained from BD (Basel). Cells expressing Ly6G/GR1 and CD11b are termed polymorphnuclear leucocytes (PMN). FSC/SSC gates were used to exclude debris in organ homogenates. Dihydrorhodamine 123 (DHR, Sigma) was used for measuring NADPH oxidase activity by measuring cellular fluorescence in FL1 channel (emission 534 nm). A fixed number of fluoroescent APC beads were used to measure cell number per sample volume and have been described before (31). α-GR1 (NimpR14) hybridoma was a generous gift from Tacchini-Cottier (Epalinges, Switzerland) (20). Macrophages were defined as $CD11b^+Ly6G^-$ cells. Neutrophils were defined as $Ly6G^+CD11b^+$ cells.

Histology. Histological samples were snap-frozen in Hanks medium and stained with anti-L.m. rabbit serum (a gift from Prof J. Bille, Lausanne), Gr-1 (PharMingen) or F4/80 (BM8, BMA) antibodies. Staining was developed using a goat anti-rat antibody (Caltag Laboratories) or goat anti-rabbit (Jackson Immuno Research) and an alkaline phosphatase-coupled donkey anti-goat antibody (Jackson Immuno Research) with naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid 2-methoxy anilide) phosphate and new fuchsin as a substrate. The presence of alkaline phosphatase activity yielded a red reaction product. The sections were counterstained with hemalum.

Determination of Cytokine Production. Serum G-CSF was measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Statistical Analysis. In all figures, unpaired two-sided *t* test or one-way analysis of variance were used where applicable. Values of $P > 0.05$ were considered nonsignificant. *P* values are as follows: * $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$.

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