Mast Cell-Derived TNF Can Exacerbate Mortality during Severe Bacterial Infections in C57BL/6-Kit^{W-sh/W-sh} Mice

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We used mast cell-engrafted genetically mast cell-deficient C57BL/6-Kit^{W-sh/W-sh} mice to investigate the roles of mast cells and mast cell-derived tumor necrosis factor in two models of severe bacterial infection. In these mice, we confirmed findings derived from studies of mast cell-deficient WBB6F₁-Kit^{W/W-v} mice indicating that mast cells can promote survival in cecal ligation and puncture (CLP) of moderate severity. However, we found that the beneficial role of mast cells in this setting can occur independently of mast cell-derived tumor necrosis factor. By contrast, using mast cell-engrafted C57BL/6-Kit^{W-sh/W-sh} mice, we found that mast cell-derived tumor necrosis factor can increase mortality during severe CLP and can also enhance bacterial growth and hasten death after intraperitoneal inoculation of *Salmonella typhimurium*. In WBB6F₁-Kit^{W-sh/W-sh} mice, mast cells enhanced survival during moderately severe CLP but did not significantly change the survival observed in severe CLP. Our findings in three types of genetically mast cell-deficient mice thus support the hypothesis that, depending on the circumstances (including mouse strain background, the nature of the mutation resulting in a mast cell deficiency, and type and severity of infection), mast cells can have either no detectable effect or opposite effects on survival during bacterial infections, eg, promoting survival during moderately severe CLP associated with low mortality but, in C57BL/

6-Kit^{W-sh/W-sh} mice, increasing mortality during severe CLP or infection with *S. typhimurium*. (Am J Pathol 2010, 176:926–938; DOI: 10.2353/ajpath.2010.090342)

The factors determining whether particular infections will be successfully controlled or progress to death are incompletely understood. Studies conducted using genetically mast cell-deficient (WB/ReJ × C57BL/6)F₁-Kit^{W/W-v} mice (WBB6F₁-Kit^{W/W-v} mice), the corresponding normal (WBB6F₁-Kit^{+/+}) mice, and mast cell-engrafted WBB6F₁-Kit^{W/W-v} mice have indicated that mast cells can increase survival during various models of bacterial infection of moderate severity,^{1–13} defined herein as infections that result in relatively low mortality in normal mice. Although mast cells can contribute to host defense against bacteria by multiple direct and indirect mechanisms,^{1–5,7–13} several groups have focused on the potential role of mast cell-derived tumor necrosis factor (TNF) in such settings.^{1–5,7} Results obtained in work using TNF-deficient mice⁴ or mice in which the actions of TNF are blocked by neutralizing antibodies^{1,2} have clearly demonstrated that TNF can have protective functions during some bacterial infections, and that such TNF-dependent effects may include the enhancement of neutrophil recruitment and/or function and the promotion of bacterial clearance.

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However, such genetic or neutralizing antibody-based approaches eliminate or reduce the function of TNF derived from all cellular sources, not just mast cell-derived TNF. Thus, there has been no direct evidence yet published that shows that mast cells represent a critical source of TNF in such settings. Moreover, WBB6F₁-*Kit*^{W/W^v} mice have a modest deficiency in neutrophils,^{14–16} and this abnormality, as well as their virtual lack of mast cells, might contribute to the increased pathology observed in these mice during certain models of bacterial infection. Therefore, in the present study, we used genetically mast cell-deficient C57BL/6-*Kit*^{W-sh/W-sh} mice (produced as in ref.¹⁷) engrafted with either wild-type mast cells or mast cells unable to produce TNF to re-examine the roles of mast cells, and mast cell-derived TNF, in two different models of bacterial infection.

Adult C57BL/6-*Kit*^{W-sh/W-sh} mice are profoundly mast cell-deficient,^{17–19} as are WBB6F₁-*Kit*^{W/W^v} mice,^{20,21} but, unlike WBB6F₁-*Kit*^{W/W^v} mice, C57BL/6-*Kit*^{W-sh/W-sh} mice have been reported to have increased levels of blood and bone marrow neutrophils.^{15,16} We used both WBB6F₁-*Kit*^{W/W^v} mice and C57BL/6-*Kit*^{W-sh/W-sh} mice to investigate the role of mast cells in moderately severe versus severe cecal ligation and puncture (CLP), with moderately severe versus severe CLP defined herein as models of CLP in which no more than 50% (in the moderately severe CLP model) vs. more than 50% (in the severe CLP model) of normal mice succumb during the first 4 days after surgery to induce acute bacterial peritonitis. Our observations in C57BL/6-*Kit*^{W-sh/W-sh} mice confirm work in WBB6F₁-*Kit*^{W/W^v} mice^{1,3–6,8} in providing evidence that mast cells can enhance host resistance and survival during moderately severe CLP, a well known model of mouse bacterial peritonitis, but provide evidence that mast cells can enhance survival in this model independently of their ability to produce TNF. By contrast, mast cell-derived TNF increased mortality during severe CLP in C57BL/6-*Kit*^{W-sh/W-sh} mice. We found that mast cell-derived TNF also can hasten mortality in C57BL/6-*Kit*^{W-sh/W-sh} mice subjected to a different model of severe bacterial infection: that induced by the intraperitoneal inoculation of *Salmonella typhimurium*.

Materials and Methods

Mice

c-kit mutant genetically mast cell-deficient (WB/ReJ-*Kit*^{W/+} × C57BL/6J-*Kit*^{W/+}) F₁-*Kit*^{W/W^v} (WBB6F₁-*Kit*^{W/W^v} or *Kit*^{W/W^v}) mice and the congenic normal WBB6F₁^{+/+} (WBB6F₁-*Kit*^{+/+}) mice, and C57BL/6J (B6J) mice, were purchased from Jackson Laboratories (Bar Harbor, ME). The *W^{sh}* or “sash” mutation was named after the broad white belt or sash observed on the coat of a single female offspring of a cross between two inbred strains (C3H/HeH × 101/H).²² *W^{sh}* homozygotes were characterized as black-eyed white mice (some of them with patches of pigment on the ear pinnae and/or base of the tail) that were viable, non-anemic and fertile,²² but which had a marked reduction in mast cells in multiple anatomical sites.²³ Genetically mast

cell-deficient C57BL/6-*Kit*^{W-sh/+} mice (B6-*Kit*^{W-sh/+} mice)²⁴ were the generous gift of Peter Besmer (Molecular Biology Program, Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, NY); while these mice were reported to be on the C57BL/6 background, it is not clear from the publication whether, and if so how many times, these mice had been backcrossed to mice from the Jackson Laboratories' C57BL/6J population. We used such B6-*Kit*^{W-sh/+} mice to produce B6-*Kit*^{W-sh/W-sh} mice for some of our initial CLP experiments; for these experiments, we used the B6-*Kit*^{+/+} littermates as wild-type controls. In addition, we backcrossed B6-*Kit*^{W-sh/+} mice with C57BL/6J (B6J) mice for 11 generations to produce B6J-*Kit*^{W-sh/W-sh} mice; for CLP experiments using these mice, we used B6J-*Kit*^{+/+} mice purchased from Jackson Laboratories as wild-type controls. Like B6-*Kit*^{W-sh/W-sh} mice,^{17,19} we found that adult (~12-week-old) B6J-*Kit*^{W-sh/W-sh} mice lacked any detectable mast cells in the peritoneal cavity or mesentery. Based on reverse transcription-PCR analysis and sequencing of the resultant PCR products, we confirmed that the *W^{sh}* inversion 3' breakpoint in our B6J-*Kit*^{W-sh/W-sh} mice and that in the B6-*Kit*^{W-sh/W-sh} mice that were characterized by Nigrovic et al¹⁶ are identical (data not shown). To generate WB/ReJ-*Kit*^{W-sh/+} mice, B6J-*Kit*^{W-sh/+} mice were crossed with WB/ReJ-*Kit*^{+/+} mice for 11 generations. Some genetically mast cell-deficient B6J-*Kit*^{W-sh/+} mice were then crossed with WB/ReJ-*Kit*^{W-sh/+} mice to produce WBB6F₁-*Kit*^{W-sh/W-sh} mice (WB/ReJ-*Kit*^{W-sh/+} × B6-*Kit*^{W-sh/+}) F₁-*Kit*^{W-sh/W-sh} and the normal wild-type littermates (ie, WBB6F₁-*Kit*^{+/+} mice). TNF-deficient mice (*Tnf*^{-/-}) on the C57BL/6J background²⁵ were bred and maintained at the Stanford University Research Animal Facility. Unless specified otherwise, all experiments were performed using 12-week-old male mice. All animal care and experimentation was conducted in accord with current National Institutes of Health guidelines and with the approval of the Stanford University Institutional Animal Care and Use Committee.

Mast Cell Engraftment of Mast Cell-Deficient Mice

Some WBB6F₁-*Kit*^{W/W^v}, B6-*Kit*^{W-sh/W-sh}, and WBB6F₁-*Kit*^{W-sh/W-sh} mice (4 to 6 weeks old) were repaired of their mast cell deficiency selectively and locally by the i.p. injection of growth factor-dependent, congenic mouse bone marrow-derived cultured mast cells (BMCMCs). Briefly, femoral bone marrow cells from WBB6F₁-*Kit*^{+/+} or B6-*Kit*^{+/+} mice were maintained *in vitro* for ~4 weeks in interleukin-3 (Peprotech, Rocky Hill, NJ)-containing medium until mast cells represented >95% of the total cells according to staining with May-Grünwald-Giemsa. 2.0 × 10⁶ BMCMCs, in 200 μl of PBS, were injected i.p. (via a 26G needle) and the mice were used for experiments, together with strain-, gender-, and age-matched mast cell-deficient mice, 4 to 6 weeks after adoptive transfer of BMCMCs. Other B6-*Kit*^{W-sh/W-sh} mice received, 4 to 6 weeks before CLP, 2.0 × 10⁶ BMCMCs generated from B6-*Tnf*^{-/-} mice. The numbers of peritoneal mast cells were

similar in C57BL/6-*Kit^{W-sh/W-sh}* mice that had been engrafted with either wild-type or TNF-deficient mast cells ($3.8 \pm 0.8\%$ or $3.6 \pm 0.7\%$ of total cells) or in WBB6F₁-*Kit^{W-sh/W-sh}* mice that had been engrafted with WBB6F₁ wild-type mast cells ($6.1 \pm 0.5\%$ of total cells), as was the distribution of mast cells in the mesentery (ie, mesenteric windows) of these three groups of mice (6.2 ± 0.96 , 6.4 ± 0.64 , or 6.1 ± 0.55 mast cells/mm², respectively).

Infection with *S. typhimurium*

The wild-type virulent *S. typhimurium* strain, SL1344, was obtained from Bruce Stocker (deceased) of Stanford University and labeled with the Lux operon to create bioluminescent SL1344/*lux* (SMB500) as reported.²⁶ Bacteria were grown as previously described²⁷ and mice were infected by i.p. injection of 25 colony forming units (CFUs) of the labeled (ie, bioluminescent) bacteria.

In Vivo Bioluminescence Imaging

Mice were imaged as previously described.²⁷ Briefly, mice were placed in an *in vivo* Imaging System (IVIS, a Xenogen product from Caliper LifeSciences, Alameda, CA). A grayscale reference image was taken under low light illumination followed by a 5 minutes image, in complete dark, of the bioluminescent signal originating from the labeled bacteria within the animals. The data were expressed as photons emitted per mouse, or region of interest, per second. A pseudocolor representation of the photon collection (red signifying most intense and blue signifying least intense) was superimposed over the grayscale reference image. Acquisition and analysis of data were performed using the LivingImage (a Xenogen product from Caliper LifeSciences) software, which runs as an overlay on the IgorPro image analysis package (Wavemetrics, Lake Oswego, OR). Mice were imaged before and at selected time points after infection.

CLP

CLP was performed as described.¹ Briefly, mice were deeply anesthetized by i.m. injection of 100 mg/kg ketamine and 20 mg/kg xylazine. The cecum was exposed by a 1- to 2-cm midline incision on the anterior abdomen and subjected to ligation of its distal portion and a single needle puncture of the ligated segment. Protocols for eliciting severe or moderately severe CLP were developed so that >50% or 20% to 50% of the wild-type mice died within 4 days after CLP. We used survival as the criterion to classify the severity of CLP, identifying procedures of performing "moderately severe" or "severe" CLP, which resulted in the death of no more than 50% or >50% of wild-type mice, respectively. However, we found, as expected, that the hypothermia induced in wild-type mice by CLP was more pronounced in mice subjected to severe CLP than in mice subjected to moderately severe CLP (a reduction in body temperature of $4.6 \pm 0.86^\circ\text{C}$ vs. $1.6 \pm 0.48^\circ\text{C}$ [mean $\pm$ SD] for severe vs.

moderately severe CLP, $P < 0.05$, $n = 5/\text{group}$) at 18 to 24 hours after surgery.

For severe CLP, we ligated the distal half or two thirds of the cecum and made a single puncture with a 22G needle; for moderately severe CLP, we ligated the distal half of the cecum and made a single puncture with a 22G needle. The cecum was then replaced into the abdomen, 1 ml of sterile saline (pyrogen-free 0.9% NaCl) was administered into the peritoneal cavity, and the incision was closed using 9-mm steel wound clips. Mice were observed for mortality at least four times daily. Mice that were clearly moribund were euthanized by CO₂ inhalation.

Quantification of Leukocytes and Platelets

Absolute numbers of neutrophils and platelets in the blood were counted using the Abbott Cell-Dyn 3500 automated hematology analyzer. Neutrophils (Gr-1^{high}/F4/80⁻ cells) in the bone marrow, spleen and peritoneal fluid, macrophages (Gr-1⁻/F4/80⁺ cells) in the peritoneal fluid, and basophils (FcεRI⁺/c-Kit⁻/DX5⁺ cells) in the blood were analyzed by flow cytometry. Briefly, red cells were lysed with AKC lysis buffer for 5 minutes. Total cell numbers were counted using a hemocytometer. Cells were blocked with unconjugated anti-CD16/CD32 on ice for 5 minutes and then stained with a combination of fluorescein isothiocyanate-labeled anti-Gr-1 (RB6-8C5, 2.5 μg/ml) and allophycocyanin-labeled anti-F4/80 (BM8, 4 μg/ml of each) antibodies (eBioscience San Diego, CA) on ice for 15 minutes for the detection of neutrophils and macrophages. To identify basophils (as c-Kit⁻CD49b⁺FcεRI α chain⁺ cells²⁸), we used a combination of phycoerythrin-labeled anti-c-Kit (2B8, 1 μg/ml) (Pharmingen, San Jose CA), allophycocyanin-labeled anti-CD49b (DX5, 8 μg/ml), and fluorescein isothiocyanate-labeled anti-FcεRI α chain (MAR-1, 2.5 μg/ml) (e-Bioscience) antibodies. The expression of cell surface markers was analyzed on a FACSCaliber (BD Biosciences) and using FlowJo Software (version 5.7.2). Gates for subpopulations of cells were based on unstained cells, as well as single color stain of the cells, to determine compensation and nonspecific fluorescence. Propidium iodide was used to detect dead cells. Only cells negative for propidium iodide were used for analysis.

Quantification of Bacterial CFUs

Dilutions of peritoneal fluids were performed and samples plated on Luria Bertani (LB) agar. Colonies were counted after overnight incubation at 37°C.

Measurement of TNF

TNF levels in the supernatants were assessed by enzyme-linked immunosorbent assay (BD OptEIA ELISA [BD Biosciences]). The detection limit for this assay is 15 pg/ml.

Figure 1. Mast cells enhance survival in WBB6F₁-Kit^{W/W-v} mice after moderately severe but not severe CLP. **A:** Survival after moderately severe CLP (50% ligation; single puncture with 22G needle) in wild-type (WBB6F₁-Kit^{+/+}) mice (black squares) ($n = 8$), WBB6F₁-Kit^{W/W-v} mast cell-deficient mice (white squares) ($n = 13$) and WBB6F₁-Kit^{+/+} BMCMCs→WBB6F₁-Kit^{W/W-v} mice (black circles) ($n = 10$). **B:** Survival after severe CLP (2/3 ligation; single puncture with 22G needle) in wild-type (WBB6F₁-Kit^{+/+}) mice (black squares) ($n = 7$), WBB6F₁-Kit^{W/W-v} mast cell-deficient mice (white squares) ($n = 10$) and WBB6F₁-Kit^{+/+} BMCMCs→WBB6F₁-Kit^{W/W-v} mice (black circles) ($n = 10$). * $P < 0.05$, ** $P < 0.01$ versus Kit^{W/W-v} mice; § $P = 0.076$, † $P < 0.05$, ††† $P < 0.001$ versus corresponding results for moderately severe CLP. All mice were females that were 12 weeks old at the beginning of the experiment. n.s., not significant.

Reverse Transcription-PCR Analysis

The Kit^{W-sh} inversion mutation in our B6J-Kit^{W-sh/W-sh} mice was assessed by PCR using PCR primers with the sequences described by Nigrovic et al¹⁶ (which flank the reported Kit^{W-sh} inversion 3' breakpoint).

Statistical Analysis

We assessed differences in the survival rates after CLP using the Mantel-Haenszel log-rank test. All other data were analyzed for statistical significance using the Mann Whitney U-test. $P < 0.05$ is considered statistically significant. Unless otherwise specified, all data are presented as mean $\pm$ SEM.

Results

Increased Mortality of Mast Cell-Deficient WBB6F₁-Kit^{W/W-v} Mice after Severe CLP Is Not Reduced by Mast Cell Engraftment

When we subjected WBB6F₁ mice to moderately severe CLP, we confirmed findings first reported by Echtenacher et al¹: the survival of genetically mast cell-deficient WBB6F₁-Kit^{W/W-v} mice (15% at day 7) was significantly worse than that of the corresponding wild-type mice (50% survival at day 7; $P = 0.0072$) or WBB6F₁-Kit^{W/W-v} mice that had been selectively engrafted with wild-type bone marrow-derived cultured mast cells (ie, Kit^{+/+} BMCMCs→Kit^{W/W-v} mice) via i.p. transfer (60% survival at day 7, $P = 0.017$) (Figure 1A). Mast cell-deficient WBB6F₁-Kit^{W/W-v} mice also exhibited higher mortality than the corresponding wild-type mice in a severe model of CLP (0 vs. 29% survival at days 3 or 7 after CLP, respectively, $P = 0.032$) (Figure 1B). However, the survival of Kit^{+/+} BMCMCs→Kit^{W/W-v} mice subjected to severe CLP was similar to that observed in mast cell-deficient

Kit^{W/W-v} mice ($P = 0.62$), suggesting that the reduced survival of WBB6F₁-Kit^{W/W-v} mice in this model may have reflected c-kit-related abnormalities other than, or in addition to, their mast cell deficiency.

These results are concordant with those of our previous study of a different model of a severe bacterial infection, that induced by the intraperitoneal injection of *S. typhimurium*.²⁷ In that study, we found that mast cell-deficient WBB6F₁-Kit^{W/W-v} mice exhibited significantly more bacterial CFUs per spleen at day 6 of *S. typhimurium* infection, as well as decreased survival, as compared with the corresponding wild-type mice.²⁷ However, the abnormalities in the response of WBB6F₁-Kit^{W/W-v} mice were not corrected in Kit^{+/+} BMCMCs→Kit^{W/W-v} mice, indicating that c-kit related factors in WBB6F₁-Kit^{W/W-v} mice other than the lack of mast cells, perhaps including defects in their neutrophil responses,¹⁴⁻¹⁶ may have contributed to their reduced survival in this model.

Mast Cells Can Enhance Survival after Moderately Severe CLP Independently of Their Ability to Produce TNF

We also investigated the role of mast cells in moderately severe versus severe CLP using C57BL/6-Kit^{W-sh/W-sh} mice. Some experiments assessing moderately severe or severe CLP were performed with B6-Kit^{W-sh/W-sh} mice and others using B6J-Kit^{W-sh/W-sh} mice; the results obtained with each of these groups were very similar and have been combined for presentation in the Figures. In contrast to mast cell-deficient WBB6F₁-Kit^{W/W-v} mice, mast cell-deficient B6-Kit^{W-sh/W-sh} or B6J-Kit^{W-sh/W-sh} mice (which we will refer to here as C57BL/6-Kit^{W-sh/W-sh} mice) have increased numbers of neutrophils (identified in flow cytometry as Gr1^{hi}, F4/80⁻ cells) in the blood, bone marrow, and spleen (^{15,16} and Supplemental Figure S1 at <http://ajp.amjpathol.org>). Moreover, since these mice have the C57BL/6 background, they can be en-

Figure 2. Mast cells, but not mast cell-derived TNF, contribute to intraperitoneal neutrophil numbers, bacterial clearance, and survival after moderately severe CLP in C57BL/6-*Kit*^{W-sh/W-sh} mice. **A:** Survival after moderately severe CLP (50% ligation; single puncture with 22G needle) in wild-type C57BL/6 (*B6-Kit*^{+/+}) mice (black squares) (*n* = 14), *B6-Kit*^{W-sh/W-sh} mast cell-deficient mice (white squares) (*n* = 15). **B–E:** Numbers of neutrophils (polymorphonuclear leukocytes [PMN]) (**B**) amount of TNF (**C**), and numbers of bacterial CFUs (**D**), in the peritoneal lavage fluid at 18 to 24 hours after moderately severe CLP in wild-type C57BL/6 mice (*B6-Kit*^{+/+}) (*n* = 5 to 7), *B6-Kit*^{W-sh/W-sh} mast cell-deficient mice (*n* = 5 to 7), *B6-Tnf*^{+/+} BMCMCs→*B6-Kit*^{W-sh/W-sh} mice (*n* = 8 to 13), and *B6-Tnf*^{-/-} BMCMCs→*B6-Kit*^{W-sh/W-sh} mice (*n* = 5 to 7). In (**A**), **P* < 0.05 versus *B6* mice; in (**B–D**), **P* < 0.05, ***P* < 0.01 for the comparisons indicated. All mice were males that were 12 weeks old at the beginning of the experiment. n.s., not significant.

grafted with wild-type or TNF-deficient mast cells of the C57BL/6 strain background to assess the extent to which TNF derived from mast cells as opposed to other cell types contributes to the outcome of moderately severe and severe CLP. This may be an important consideration, as evidence has been reported indicating that mast cells from mice of different strain backgrounds can differ in their expression of cell surface structures²⁹ and in their responses to activation via FcεRI.³⁰

Like WBB6F₁-*Kit*^{W-sh/W-sh} mice, mast cell-deficient C57BL/6-*Kit*^{W-sh/W-sh} mice exhibited reduced survival after moderately severe CLP when compared with the congenic normal mice (16 vs. 61% survival at day 7, respectively, *P* = 0.028) (Figure 2A). However, there were no statistically significant differences in survival after moderately severe CLP for wild-type mice or C57BL/6-*Kit*^{W-sh/W-sh} mice that had been engrafted with either wild-type (*Tnf*^{+/+}) mast cells (*Tnf*^{+/+} BMCMCs→*Kit*^{W-sh/W-sh} mice) or TNF-deficient (*Tnf*^{-/-}) mast cells (*Tnf*^{-/-} BMCMCs→*Kit*^{W-sh/W-sh} mice) (Figure 2A). These results therefore do not support the hypothe-

sis that mast cells have to be able to produce TNF to enhance survival after moderately severe CLP.

Several lines of evidence indicate that one mechanism by which mast cells can augment innate immunity during bacterial infections is by initiating and augmenting leukocyte recruitment, thereby enhancing pathogen clearance.^{1,2,12,31,32} At 18 to 24 hours after moderately severe CLP, C57BL/6-*Kit*^{W-sh/W-sh} mice, which had been engrafted with either wild-type or TNF-deficient mast cells, contained increased numbers of peritoneal neutrophils (Figure 2B) as compared with mast cell-deficient C57BL/6-*Kit*^{W-sh/W-sh} mice. Although all groups of mice exhibited very similar amounts of TNF in the peritoneal cavity at 18 to 24 hours after CLP (Figure 2C), only mast cell-deficient C57BL/6-*Kit*^{W-sh/W-sh} mice (the group with the lowest levels of intraperitoneal neutrophils [Figure 2C]) exhibited high numbers of intraperitoneal bacterial CFUs at that time (Figure 2D). These results suggest that mast cells can have effects that increase numbers of intraperitoneal neutrophils, and bacterial clearance, in

Figure 3. Mast cells, and mast cell-derived TNF, increase intraperitoneal neutrophil numbers and amounts of intraperitoneal TNF, but reduce survival, after severe CLP in C57BL/6-Kit^{W-sh/W-sh} mice. **A:** Survival after severe CLP (2/3 ligation; single puncture with 22G needle) in wild-type C57BL/6 mice (B6-Kit^{+/+}) (black squares) (*n* = 19), B6-Kit^{W-sh/W-sh} mast cell-deficient mice (white squares) (*n* = 23), B6-Tnf^{+/+} BMCMCs→B6-Kit^{W-sh/W-sh} mice (blue circles) (*n* = 20) and B6-Tnf^{-/-} BMCMCs→B6-Kit^{W-sh/W-sh} mice (yellow rhomboids) (*n* = 20). **B–D:** Numbers of neutrophils (polymorphonuclear leukocytes [PMN]) (**B**), amount of TNF (**C**), and numbers of bacterial CFUs (**D**), in the peritoneal lavage fluid at 18 to 24 hours after severe CLP in wild-type C57BL/6 mice (B6-Kit^{+/+}) (*n* = 7 to 11), B6-Kit^{W-sh/W-sh} mast cell-deficient mice (*n* = 7 to 16), B6-Tnf^{+/+} BMCMCs→B6-Kit^{W-sh/W-sh} mice (*n* = 9 to 13), and B6-Tnf^{-/-} BMCMCs→B6-Kit^{W-sh/W-sh} mice (*n* = 5 to 8). In (**A**), **P* < 0.05 versus B6-Kit^{W-sh/W-sh} mice; §*P* < 0.01 vs. B6-Kit^{+/+} mice or B6-Tnf^{+/+} BMCMCs→B6-Kit^{W-sh/W-sh} mice; in (**B–D**), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 for the comparisons indicated; †*P* < 0.05, ††*P* < 0.01, †††*P* < 0.001, ††††*P* < 0.0001 versus corresponding results for moderately severe CLP. Survival curves for B6-Kit^{W-sh/W-sh} mast cell-deficient mice and B6-Tnf^{-/-} BMCMCs→B6-Kit^{W-sh/W-sh} mice were *P* = 0.17 and *P* = 0.11 versus corresponding results for moderately severe CLP. All mice were males that were 12 weeks old at the beginning of the experiment. n.s., not significant.

this model independently of the mast cell's ability to influence levels of TNF.

It has been reported that in some models of intraperitoneal inflammation,^{2,33} mast cells represent an early source of TNF. We therefore also measured TNF levels in the peritoneum of wild-type C57BL/6 mice versus mast cell-deficient C57BL/6-Kit^{W-sh/W-sh} mice at 6 hours after moderately severe CLP. We found that while the levels of intraperitoneal TNF were substantially lower in both groups than when measured at 18 to 24 hours after CLP (compare results in Figure 2C and Supplemental Figure S1A at <http://ajp.amjpathol.org>), the levels in the mast cell-deficient C57BL/6-Kit^{W-sh/W-sh} mice were only slightly (but not significantly) lower than those in the wild-type mice. Similarly, we did not observe significant differences

in the numbers of neutrophils in the peritoneal cavity of wild-type (C57BL/6-Kit^{+/+}) vs. C57BL/6-Kit^{W-sh/W-sh} mast cell deficient mice at 6 hours after moderately severe CLP (Supplemental Figure S1B at <http://ajp.amjpathol.org>). While these data do not rule out the possibility that mast cells may represent one source of "early TNF" in the moderately severe CLP model in C57BL/6 mice, they do indicate that any such effects are relatively modest in this setting.

Mast Cell-Derived TNF Can Impair Survival after Severe CLP in C57BL/6-Kit^{W-sh/W-sh} Mice

In contrast to our findings in WBB6F₁-Kit^{W/W-v} mice (Figure 1B), mast cell-deficient C57BL/6-Kit^{W-sh/W-sh} mice

exhibited significantly better survival than the corresponding wild-type mice after severe CLP ($P = 0.029$) (Figure 3A). The survival of $Tnf^{+/+}$ BMCMCs $\rightarrow Kit^{W-sh/W-sh}$ mice was not significantly different from that of C57BL/6 wild-type mice ($P = 0.25$) (Figure 3A), and the survival of $Tnf^{-/-}$ BMCMCs $\rightarrow Kit^{W-sh/W-sh}$ mice was not significantly different from that of mast cell-deficient C57BL/6- $Kit^{W-sh/W-sh}$ mice ($P = 0.45$). Overall, these data show that wild-type mast cells, but not TNF-deficient mast cells, can significantly impair the survival of C57BL/6- $Kit^{W-sh/W-sh}$ mice subjected to severe CLP.

When assessed at 18 to 24 hours after severe CLP, C57BL/6- $Kit^{W-sh/W-sh}$ mice engrafted with wild-type mast cells exhibited significantly higher numbers of neutrophils (Figure 3B) and amounts of TNF (Figure 3C) in the peritoneal cavity than did C57BL/6- $Kit^{W-sh/W-sh}$ mice engrafted with $Tnf^{-/-}$ mast cells; numbers of bacterial CFUs were also higher, although this result was not statistically significant ($P = 0.21$) (Figure 3D). The two groups with the worst survival after severe CLP (ie, wild-type mice and C57BL/6- $Kit^{W-sh/W-sh}$ mice engrafted with wild-type mast cells) also had significantly higher numbers of neutrophils, amounts of TNF and numbers of bacterial CFUs in the peritoneal cavity than did the corresponding groups subjected to moderately severe CLP (compare Figure 2, B–D with Figure 3, B–D). By contrast, the two groups with the best survival after severe CLP (ie, mast cell-deficient C57BL/6- $Kit^{W-sh/W-sh}$ mice and C57BL/6- $Kit^{W-sh/W-sh}$ mice engrafted with $Tnf^{-/-}$ mast cells) had amounts of TNF and numbers of bacterial CFUs in the peritoneal cavity 18 to 24 hours after severe CLP that did not differ significantly from the corresponding values in the same groups of mice during moderately severe CLP (compare Figure 2, C and D, with Figure 3, C and D).

Our findings demonstrate that, in C57BL/6- $Kit^{W-sh/W-sh}$ mice, mast cells have effects that can significantly increase levels of TNF during severe CLP (whether by producing the TNF themselves or by influencing the ability of other cells to do so), but not during moderately severe CLP (compare Figure 2C with Figure 3C). Our data also show that such effects are associated with (and possibly may contribute to) higher levels of neutrophil recruitment/retention in the severe CLP model, even though mast cell-derived TNF does not appear to contribute to the mast cell-dependent increase in intraperitoneal neutrophils during moderately severe CLP (compare Figure 2B with Figure 3B).

In WBB6F₁-Kit^{W-sh/W-sh} Mice, Mast Cells Can Significantly Enhance Survival in Moderately Severe CLP but Not in Severe CLP

To assess the extent to which mouse strain background might influence differences in responses to CLP in WBB6F₁- $Kit^{W-sh/W-sh}$ versus C57BL/6- $Kit^{W-sh/W-sh}$ mice, we generated WBB6F₁- $Kit^{W-sh/W-sh}$ mice. We first compared numbers of various leukocyte populations and blood platelets, and spleen size, in WBB6F₁- $Kit^{W-sh/W-sh}$ mice,

B6J- $Kit^{W-sh/W-sh}$ mice, and WBB6F₁- $Kit^{W-sh/W-sh}$ mice and the corresponding wild-type $Kit^{+/+}$ mice (Supplemental Figures 2–6 at <http://ajp.amjpathol.org>).

Like naïve B6- $Kit^{W-sh/W-sh}$ mice¹⁶ and B6J- $Kit^{W-sh/W-sh}$ mice, WBB6F₁- $Kit^{W-sh/W-sh}$ mice exhibited increased numbers of neutrophils ($Gr1^{+}/hi$, F4/80[−] cells) in the blood, bone marrow and spleen (see Supplemental Figure S2 at <http://ajp.amjpathol.org>), increased numbers of platelets in the blood (see Supplemental Figure S3 at <http://ajp.amjpathol.org>), and an enlarged spleen (see Supplemental Figure S4 at <http://ajp.amjpathol.org>) when compared with their littermate wild-type controls. However, we observed two differences between WBB6F₁- $Kit^{W-sh/W-sh}$ mice and B6J- $Kit^{W-sh/W-sh}$ mice that probably represent effects of strain background: naïve WBB6F₁- $Kit^{W-sh/W-sh}$ mice, but not naïve B6J- $Kit^{W-sh/W-sh}$ mice, exhibited significantly increased numbers of neutrophils ($Gr1^{+}$, F4/80[−] cells) in the peritoneal cavity (see Supplemental Figure S5A at <http://ajp.amjpathol.org>), and B6J- $Kit^{W-sh/W-sh}$ mice, but not WBB6F₁- $Kit^{W-sh/W-sh}$ mice, exhibited significantly reduced numbers of $Gr1^{-}$, F4/80⁺ macrophages in the peritoneal cavity (see Supplemental Figure S5B at <http://ajp.amjpathol.org>), when compared with their age- and gender-matched wild-type controls. In contrast to WBB6F₁- $Kit^{W-sh/W-sh}$ mice, which have reduced numbers of basophils (defined here as c-Kit[−]CD49b[DX5]⁺ FcεRI α chain⁺ cells²⁸) in the blood (see Supplemental Figure S6 at <http://ajp.amjpathol.org>), we found that numbers of basophils were increased in the blood of B6J- $Kit^{W-sh/W-sh}$ mice and WBB6F₁- $Kit^{W-sh/W-sh}$ mice (see Supplemental Figure S6 at <http://ajp.amjpathol.org>). However, it is not clear whether basophils have any critical function in the CLP model.

Our results indicate that both strain background, ie, (WB/ReJ × C57BL/6)F₁ versus C57BL/6J, and the type of mutation affecting c-Kit expression (ie, $Kit^{W-sh/W-sh}$ versus $Kit^{W-sh/W-sh}$) can influence the aspects of phenotype we analyzed. Our findings also show that, for most of the phenotypic features assessed in this study, WBB6F₁- $Kit^{W-sh/W-sh}$ mice more closely resembled C57BL/6J- $Kit^{W-sh/W-sh}$ mice than WBB6F₁- $Kit^{W-sh/W-sh}$ mice.

We then investigated the responses of WBB6F₁- $Kit^{W-sh/W-sh}$ mice to moderately severe and severe CLP. Like the other mast cell-deficient mice analyzed, WBB6F₁- $Kit^{W-sh/W-sh}$ mice exhibited reduced survival after moderately severe CLP when compared with the corresponding normal mice (58 vs. 6% survival at day 7, respectively, $P = 0.001$). Engraftment of WBB6F₁- $Kit^{W-sh/W-sh}$ mice with wild-type BMCMCs improved their survival to levels that were not significantly different from those found in wild-type mice (Figure 4A). By contrast, after severe CLP (Figure 4B), WBB6F₁- $Kit^{W-sh/W-sh}$ mice exhibited slightly (but not significantly) better survival than either the littermate wild-type controls (30% vs. 17% survival at day 7, respectively, $P = 0.21$) or the WBB6F₁- $Kit^{W-sh/W-sh}$ mice that had been engrafted with wild-type mast cells (0% survival at day 7, $P = 0.64$).

All groups of mice exhibited similar numbers of neutrophils (Figure 4C), amounts of TNF (Figure 4D) and numbers of bacterial CFUs (Figure 4E) in the peritoneal cavity at 18 to 24 hours after severe CLP. However, compared with the

Figure 4. Mast cells enhance survival in WBB6F₁-Kit^{W-sh/W-sh} mice after moderately severe but not severe CLP. **A:** Survival after moderately severe CLP (50% ligation; single puncture with 22G needle) in wild-type (WBB6F₁-Kit^{+/+}) mice (black squares) (*n* = 12), WBB6F₁-Kit^{W-sh/W-sh} mast cell-deficient mice (white squares) (*n* = 16) and WBB6F₁-Kit^{+/+} BMCMCs→WBB6F₁-Kit^{W-sh/W-sh} mice (blue circles) (*n* = 9). **B:** Survival after severe CLP (2/3 ligation; single puncture with 22G needle) in wild-type (WBB6F₁-Kit^{+/+}) mice (black squares) (*n* = 30), WBB6F₁-Kit^{W-sh/W-sh} mast cell-deficient mice (white squares) (*n* = 16) and WBB6F₁-Kit^{+/+} BMCMCs→WBB6F₁-Kit^{W-sh/W-sh} mice (blue circles) (*n* = 5). **P* < 0.05, ****P* < 0.001 versus WBB6F₁-Kit^{W-sh/W-sh} mice; ††*P* < 0.01 versus corresponding results for moderately severe CLP. All mice were males that were 12 weeks old at the beginning of the experiment. n.s., not significant. **C-E:** Numbers of neutrophils (polymorphonuclear leukocytes [PMN]) (**C**), amount of TNF (**D**), and numbers of bacterial CFUs (**E**), in the peritoneal lavage fluid at 18 to 24 hours after severe CLP in wild-type (WBB6F₁-Kit^{+/+}) (*n* = 4 to 13), WBB6F₁-Kit^{W-sh/W-sh} mast cell-deficient mice (*n* = 3 to 11) and WBB6F₁-Kit^{+/+} BMCMCs→WBB6F₁-Kit^{W-sh/W-sh} mice (*n* = 3 to 10). n.s., not significant.

corresponding groups of mice on the C57BL/6 background, WBB6F₁ wild-type mice and WBB6F₁-Kit^{W-sh/W-sh} mice engrafted with wild-type mast cells exhibited significantly lower numbers of neutrophils (compare Figures 3B and 4C), amounts of TNF (compare Figures 3C and 4D) and bacterial CFUs (compare Figures 3D and 4E) in the peritoneal cavity at 18 to 24 hours after severe CLP. These observations support the conclusion that C57BL/6 mice exhibit a more pronounced

inflammatory response to severe CLP than do WBB6F₁ mice, at least as judged by levels of TNF and neutrophils in the peritoneal cavity. The differences in the findings in severe CLP in wild-type C57BL/6 vs. WBB6F₁ mice also are consistent with the possibility that strain differences affecting mast cells result in mast cells having a more substantial effect in enhancing inflammation, and reducing survival, after severe CLP in C57BL/6 mice than in the WBB6F₁ animals.

Figure 5. Mast cells impair innate immunity in C57BL/6-Kit^{W-sh/W-sh} mice during infection with *S. typhimurium*. (A and B) Survival and (C), bioluminescent signals at 5 days in mice that received 25 CFU *S. typhimurium* SL1344*lux* via an i.p. injection; wild-type C57BL/6J mice (B6-Kit^{+/+}, black squares) (*n* = 18), mast cell-deficient C57BL/6-Kit^{W-sh/W-sh} mice (B6-Kit^{W-sh/W-sh}, white squares, the same data are shown in A and B for clarity) (*n* = 21), wild-type B6-Tnf^{+/+} BMCMCs→B6-Kit^{W-sh/W-sh} mice (blue circles) (*n* = 26) and B6-Tnf^{-/-} BMCMCs→B6-Kit^{W-sh/W-sh} mice (yellow rhomboids) (*n* = 14). In C, the intensity of bioluminescence is depicted using a pseudocolor scale superimposed over a grayscale reference image of the mice. Results in (C) are representative of the similar results obtained in 3 to 4 separate experiments. In (A), **P* < 0.05 versus B6 mice; in (B), **P* < 0.05, ****P* < 0.0001 versus B6-Tnf^{+/+} BMCMCs→B6-Kit^{W-sh/W-sh} mice. All mice were males that were 12 weeks old at the beginning of the experiment.

Mast Cell-Derived TNF Can Contribute to Bacterial Proliferation and Hasten Death during Infection with *S. typhimurium*

Our findings in mice that were subjected to severe CLP were unexpected, since prior work indicated that mast cells can have protective effects in moderately severe CLP and in other models of bacterial infection.^{1-5,7-11,13} We therefore decided to examine the role of mast cells and mast cell-derived TNF in another model of severe bacterial infection.

S. typhimurium is a Gram-negative intracellular pathogen that causes a deadly systemic infection in mice that resembles typhoid fever in humans.³⁴ We induced a severe infection with *S. typhimurium* in mice by injecting a virulent strain (SL1344) of *S. typhimurium* (SL1344) into the peritoneal cavity.³⁵ In contrast to mast cell-deficient WBB6F₁-Kit^{W^{sh}/W^{sh}} mice (which we previously showed died more rapidly after i.p. inoculation with *S. typhimurium* than did the corresponding wild-type mice²⁷), mast cell-deficient C57BL/6-Kit^{W-sh/W-sh} mice exhibited slightly but significantly (*P* = 0.019) prolonged survival, compared with the corresponding wild-type mice, on i.p. injection of bioluminescent *S. typhimurium* (Figure 5A).

We then assessed whether adoptive transfer of mast cells influenced the survival of C57BL/6-Kit^{W-sh/W-sh} mice in this model. Like after severe CLP (Figure 3A), we found that C57BL/6-Kit^{W-sh/W-sh} mice selectively engrafted with wild-type (C57BL/6-Tnf^{+/+}) mast cells had significantly shorter survival after *S. typhimurium* inoculation than either mast cell-deficient C57BL/6-Kit^{W-sh/W-sh} mice or C57BL/6-Kit^{W-sh/W-sh} mice engrafted with Tnf^{-/-} BMCMCs (Figure 5B). Tnf^{+/+} BMCMCs→Kit^{W-sh/W-sh} mice also exhibited stronger bioluminescent signals on d 5 of infection, reflecting greater numbers of bioluminescent *S. typhimurium*, than did either mast cell-deficient C57BL/6-Kit^{W-sh/W-sh} mice or C57BL/6-Kit^{W-sh/W-sh} mice engrafted with Tnf^{-/-} BMCMCs (Figure 5C).

However, when assessed at 18 to 24 hours after *S. typhimurium* inoculation, C57BL/6-Kit^{W-sh/W-sh} mice engrafted with wild-type mast cells exhibited significantly lower numbers of neutrophils in the peritoneal cavity than either mast cell-deficient C57BL/6-Kit^{W-sh/W-sh} mice or C57BL/6-Kit^{W-sh/W-sh} mice engrafted with Tnf^{-/-} mast cells (Supplemental Figure S7 at <http://ajp.amjpathol.org>). This finding is the opposite of that obtained when these groups of mice were subjected to the severe model of CLP (see Figure 3B). Moreover, when we analyzed the

same mice shown in Supplemental Figure S7 (<http://ajp.amjpathol.org>) for intraperitoneal levels of TNF, we found that no TNF was detectable in any of the mice at 18 to 24 hours after *S. typhimurium* inoculation. Thus, this model of *S. typhimurium* infection is associated with substantially lower levels of intraperitoneal neutrophils, and TNF production, than our models of CLP.

These results show that TNF production by mast cells can enhance bacterial proliferation (as reflected by increased bioluminescent signals [Figure 5C]) and hasten death (Figure 5B) in this model of severe infection with *S. typhimurium* in C57BL/6-*Kit*^{W-sh/W-sh} mice. However, unlike in our model of severe CLP, in mice infected with *S. typhimurium*, the hastened death observed in C57BL/6-*Kit*^{W-sh/W-sh} mice containing wild-type mast cells was not accompanied by the presence of higher numbers of peritoneal neutrophils than in C57BL/6-*Kit*^{W-sh/W-sh} mice containing *Tnf*^{-/-} mast cells (Supplemental Figure S7 at <http://ajp.amjpathol.org>).

Discussion

Our findings show that mast cell engraftment into the peritoneal cavity of mast cell-deficient mice can have effects that range from enhancing or impairing the animals' innate immunity to bacterial infection, with the result obtained in individual experiments depending on both the details of the model of infection analyzed and the type of mast cell-deficient mice studied. Moreover, we show in C57BL/6-*Kit*^{W-sh/W-sh} mice that mast cell-derived TNF is not required for mast cells to enhance survival in one model of innate immunity, moderately severe CLP, but that mast cell-derived TNF can contribute to the deleterious effects of mast cells in severe CLP or after intraperitoneal inoculation with *S. typhimurium*.

We investigated CLP both because this model is widely used (and has been called the "gold standard") in sepsis research,³⁶ and because many studies investigating the potential roles of mast cells in innate immunity to bacterial infection have used this model.^{1,3-6,8,10,13} However, the great majority of such studies have investigated moderately severe CLP,^{1,3-6,8,10} in which most of the wild-type mice survive. In the present study, we used three different types of mast cell-engrafted, genetically mast cell-deficient mice to reinvestigate the contribution of mast cells to survival after moderately severe CLP and to analyze the role of mast cells in severe CLP, in which most wild-type mice succumb.

It has been reported that mast cells can enhance survival in WBB6F₁-*Kit*^{W/W-v} mice subjected to moderately severe CLP,^{1,3-6,8,10,13} and we have confirmed that finding (Figure 1A). Moreover, we report herein that mast cells also can enhance survival after moderately severe CLP in C57BL/6-*Kit*^{W-sh/W-sh} mice (Figure 2A) and in WBB6F₁-*Kit*^{W-sh/W-sh} mice (Figure 4A). These findings indicate that the beneficial function of mast cells on survival in this model of innate immunity to bacterial infection can be observed in mice of two different strain backgrounds (ie, WBB6F₁ and C57BL/6) and in WBB6F₁ mice with either of two distinct mutations, which result in mast cell

deficiency (ie, *Kit*^{W/W-v} and *Kit*^{W-sh/W-sh}). However, our data indicate that, during severe CLP, mast cells either can have no statistically significant effect on survival (in mast cell-engrafted WBB6F₁-*Kit*^{W/W-v} [Figure 1B] or WBB6F₁-*Kit*^{W-sh/W-sh} [Figure 4B] mice) or can actually impair survival (in C57BL/6-*Kit*^{W-sh/W-sh} mice engrafted with wild-type mast cells [Figure 3A]).

Our findings thus show that variation in mortality rates that are observed among different types of mast cell-engrafted mast cell-deficient mice subjected to severe CLP can reflect differences in their strain background, as well as differences in the consequences of the mutations that result in their mast cell deficiency. However, it bears emphasis that WBB6F₁-*Kit*^{W/W-v} and C57BL/6-*Kit*^{W-sh/W-sh} mice,¹⁴⁻²¹ as well as the WBB6F₁-*Kit*^{W-sh/W-sh} mice that were generated and characterized in the present study, exhibit a variety of consequences of their mutations in addition to a profound deficiency in mast cells. These additional abnormalities clearly must be kept in mind when interpreting the results of experiments that employ such animals to investigate mast cell functions.

For example, the phenotypic abnormalities of WBB6F₁-*Kit*^{W/W-v}, C57BL/6-*Kit*^{W-sh/W-sh}, and WBB6F₁-*Kit*^{W-sh/W-sh} mice include alterations in the numbers of hematopoietic populations other than mast cells, including neutrophils¹⁴⁻¹⁶ (Supplemental Figure S1 at <http://ajp.amjpathol.org>), macrophages¹⁶ (Supplemental Figure S4B at <http://ajp.amjpathol.org>), and basophils (Supplemental Figure S5 at <http://ajp.amjpathol.org>). Therefore, it is possible that in some biological responses the presence of adoptively transferred mast cells in genetically mast cell-deficient mice can compensate for some of the consequences of the other defects in such animals. Indeed, this has been proposed as one explanation of why some models of experimental arthritis that feature an important role for neutrophils are poorly expressed in the relatively neutrophil-deficient WBB6F₁-*Kit*^{W/W-v} mice (unless the animals contain adoptively transferred populations of mast cells), but are normally expressed in mast cell-deficient C57BL/6-*Kit*^{W-sh/W-sh} mice, which have levels of neutrophils higher than those in the corresponding wild-type mice.^{15,16}

Compensation by adoptively transferred mast cells for some consequence of the animal's relative deficiency in neutrophils may also contribute to the beneficial role of mast cells observed in mast cell-engrafted WBB6F₁-*Kit*^{W/W-v} mice subjected to moderately severe CLP. However, we showed that adoptive transfer of mast cells also increases survival after moderately severe CLP in both C57BL/6-*Kit*^{W-sh/W-sh} and WBB6F₁-*Kit*^{W-sh/W-sh} mice, which have baseline levels of neutrophils in the blood, bone marrow, spleen, and peritoneal cavity that are not reduced and in many sites are significantly increased compared with those in the corresponding wild-type mice. Taken together, this evidence shows that, in C57BL/6-*Kit*^{W-sh/W-sh} and WBB6F₁-*Kit*^{W-sh/W-sh} mice, mast cells must contribute to improved survival after moderately severe CLP by mechanisms beyond simply compensating for a defect in numbers of neutrophils.

Our studies in C57BL/6-*Kit*^{W-sh/W-sh} mice engrafted with wild-type versus TNF-deficient mast cells indi-

cated that wild-type mast cells can impair survival in C57BL/6-*Kit^{W-sh/W-sh}* mice subjected to severe CLP, and that this effect is significantly reduced if the adoptively transferred mast cell population lacks the ability to make TNF (Figure 3A). It is likely that death after CLP reflects the operation of complex processes, and additional work will be required to understand how mast cell-derived TNF impairs survival in this setting. One possibility is that the ability of the adoptively transferred wild-type mast cells to produce TNF and other mediators, when added to the already increased levels of neutrophils at baseline in C57BL/6-*Kit^{W-sh/W-sh}* mice, may result in an environment that in some ways may be more "pro-inflammatory" than that present in the corresponding wild-type animals. However, we found that the adoptive transfer of wild-type mast cells to C57BL/6-*Kit^{W-sh/W-sh}* mice appeared to allow these animals to respond to severe CLP, at least with respect to intraperitoneal levels of TNF and neutrophils, in a way that was very similar to that of the corresponding wild-type mice. Thus, even though C57BL/6-*Kit^{W-sh/W-sh}* mice had significantly more neutrophils in the blood, bone marrow and spleen at baseline than did the corresponding wild-type mice (Supplemental Figure 2), when tested 18 to 24 hours after the induction of moderately severe or severe CLP, the levels of intraperitoneal neutrophils or TNF were quite similar in C57BL/6-*Kit^{W-sh/W-sh}* mice that had been engrafted with wild-type mast cells and in the corresponding wild-type mice (Figures 2, B and C, and 3, B and C).

It has been proposed that neutrophils can express opposing roles during CLP and other innate immune response to bacterial infection, promoting bacterial clearance but, in severe infections, also contributing to organ damage.³⁷ However, C57BL/6-*Kit^{W-sh/W-sh}* mice engrafted with wild-type versus TNF-deficient mast cells may not differ only in the levels of TNF (Figure 2C) and neutrophils (Figure 2B) observed after severe CLP, but also in other mediators produced in this setting, which may be influenced by TNF and/or neutrophils. Proving which of these factors contribute most to lethality in this setting may be difficult. For example, using approaches to block the activity of TNF (which has been done by others in studies of moderately severe CLP)^{1,38} would block TNF from all sources, not just TNF of mast cell origin.

To investigate the possibility that neutrophils contribute to mortality in our model of severe CLP, we attempted to diminish neutrophils in C57BL/6J mice using both the anti-Ly-6G/Ly-6C antibody (clone RB6-8C5)³⁹ and the anti-Ly-6G antibody (clone 1A8).⁴⁰ However, in our hands, treatment of mice with either of these antibodies, which have been used to reduce neutrophils in mice *in vivo*,^{37,41} also resulted in a less dramatic, but significant, reduction in blood monocytes after severe CLP (data not shown). Moreover, we found that the administration of an isotype-matched control antibody (ie, rat IgG2a, κ , clone R35-95, catalog number 553927, BD Biosciences) to wild-type C57BL/6J mice also resulted in a significant drop in blood neutrophils, as well as a significant reduction in the mortality associated with severe CLP (data not shown). Thus, our attempts to achieve a selective and

specific reduction in neutrophils in our model of severe CLP have not yet yielded readily interpretable results. In summary, although we can conclude that the presence of wild-type (as opposed to TNF-deficient) mast cells in C57BL/6-*Kit^{W-sh/W-sh}* mice subjected to severe CLP results in these animals developing a more "pro-inflammatory" response to the infection, as judged by levels of intraperitoneal TNF and neutrophils, it is not yet clear to what extent these findings may contribute to the ability of wild-type mast cells to increase the mortality of severe CLP.

In light of the provocative finding that mast cell-derived TNF increased mortality in severe CLP in C57BL/6-*Kit^{W-sh/W-sh}* mice, we re-examined the hypothesis that mast cells can contribute to survival during moderately severe CLP in part through the production of TNF.¹ We found that, in C57BL/6-*Kit^{W-sh/W-sh}* mice, mast cell-derived TNF did not detectably contribute to the process by which mast cells enhanced survival (Figure 2A). At 18 to 24 hours after the induction of moderately severe CLP, intraperitoneal levels of neutrophils were substantially higher in C57BL/6-*Kit^{+/+}* wild-type mice or in C57BL/6-*Kit^{W-sh/W-sh}* mice engrafted with wild-type or TNF-deficient mast cells than they were in mast cell-deficient C57BL/6-*Kit^{W-sh/W-sh}* mice (Figure 2B), and the numbers of bacterial CFUs were significantly higher in the mast cell-deficient C57BL/6-*Kit^{W-sh/W-sh}* mice than in each of the other three groups (Figure 2D). However, at 18 to 24 hours after CLP, all four of these groups had very similar levels of intraperitoneal TNF (Figure 2C). C57BL/6-*Kit^{W-sh/W-sh}* mice and the corresponding wild-type mice also exhibited similar levels of intraperitoneal TNF and neutrophils 6 hours after induction of severe CLP (Supplemental Figure S1 at <http://ajp.amjpathol.org>). Taken together with the survival data, which showed that only the survival of mast cell-deficient C57BL/6-*Kit^{W-sh/W-sh}* mice was significantly worse than that of the C57BL/6-*Kit^{+/+}* wild-type mice (Figure 2A), these findings argue against a critical role for mast cell-derived TNF either in altering intraperitoneal levels of TNF or in contributing to the mast cell-dependent increases in intraperitoneal neutrophils or decreases in intraperitoneal bacterial CFUs during moderately severe CLP. Mast cells also have been reported not to influence the increased levels of intraperitoneal TNF which were observed in mice after they received an intraperitoneal injection with a thousand *Klebsiella pneumoniae* bacteria.¹²

We do not consider it surprising that mast cells can contribute to survival after moderately severe CLP even if they lack the ability to make TNF. Indeed, there is evidence that several mast cell products can enhance survival during innate immune responses to bacterial infection. In studies of moderately severe CLP, it has been reported that mast cells can enhance survival by production of interleukin-6⁴² or by the action of mast cell-associated proteases including mast cell carboxypeptidase A or neurolysin (which can enhance survival by degrading potentially toxic endogenous peptides produced during CLP, such as endothelin-1^{8,13} or neurolysin¹³). In studies of mice injected intraperitoneally with *K. pneumoniae*, survival can be enhanced by mouse mast cell protease 6.¹²

Thus, analyses of moderately severe CLP and other models of bacterial infection show that mast cells can contribute to survival in such settings via several different mechanisms. Moreover, although work by several groups (including our own) has shown that TNF also can confer benefit during CLP^{4,38} or other models of innate immune responses to bacteria^{43,44} in mice, it is well known that mast cells represent just one among many potential cellular sources of TNF.^{45,46} Thus, while we haven't ruled out an important role for mast cell-derived TNF in other models of innate immunity, our present study shows that, at least in C57BL/6 mice, mast cells are unlikely to represent a critical source of TNF during moderately severe CLP.

To extend our observations beyond models of CLP, we investigated a different model of severe bacterial infection, that induced by the intraperitoneal injection of *S. typhimurium*.²⁷ In C57BL/6-Kit^{W-sh/W-sh} mice, we found that mice engrafted with wild-type mast cells died more rapidly than those engrafted with TNF-deficient mast cells or those which virtually lacked mast cells (Figure 5). This result indicated that mast cell-derived TNF can exacerbate the pathology associated with this model of severe infection, as it did in severe CLP. However, this model of *S. typhimurium* infection was associated with substantially lower numbers of intraperitoneal neutrophils at 18 to 24 hours after the initiation of infection than the model of severe CLP (compare Figures 3B and Supplemental Figure S7 at <http://ajp.amjpathol.org>). Moreover, numbers of neutrophils at 18 to 24 hours after injection of *S. typhimurium* were significantly lower in C57BL/6-Kit^{W-sh/W-sh} mice engrafted with wild-type mast cells than in either C57BL/6-Kit^{W-sh/W-sh} mice or C57BL/6-Kit^{W-sh/W-sh} mice engrafted with TNF-deficient mast cells (Supplemental Figure S7 at <http://ajp.amjpathol.org>), and levels of intraperitoneal TNF were below the limits of detection in each of these groups.

Our findings show that mast cell-derived TNF can increase and/or hasten mortality in C57BL/6-Kit^{W-sh/W-sh} mice in two very different models of severe bacterial infection: severe CLP and intraperitoneal inoculation with *S. typhimurium*. Our findings also suggest that the detrimental effect of mast cell-derived TNF in innate immunity to bacterial infection can be observed in settings that are or are not associated with mast cell-derived TNF-dependent enhancement of the numbers of neutrophils, or levels of TNF, in the peritoneal cavity of the infected mice.

In summary, our findings show that the roles of mast cells, and mast cell-derived TNF, in innate immune responses to bacterial infection depend on the model of infection analyzed, and on the type of genetically mast cell-deficient mice used to investigate such models. In all three types of mast cell-deficient mice analyzed, mast cells significantly improved survival in mice subjected to moderately severe CLP. In C57BL/6-Kit^{W-sh/W-sh} mice, mast cells also increased levels of intraperitoneal neutrophils in this setting and improved bacterial clearance, but these effects were seen in C57BL/6-Kit^{W-sh/W-sh} mice that contained either wild-type or TNF-deficient mast cells. Our findings in the moderately severe model of CLP thus are consistent with the now widely held view that when

mast cells contribute to effective innate immune responses to infection, they do so at least in part by enhancing neutrophil responses and bacterial clearance.^{1,2,12,31,32} However, we also showed that mast cells can mediate such effects in moderately severe CLP even if they are unable to produce TNF. By contrast, we found that mast cells, and mast cell-derived TNF, can increase and/or hasten mortality in C57BL/6-Kit^{W-sh/W-sh} mice in two different models of severe bacterial infection: severe CLP and intraperitoneal inoculation with *S. typhimurium*. While the mechanisms by which mast cell-derived TNF can increase pathology in these two models remain to be defined, at 18 to 24 hours after the onset of infection, mast cell-derived TNF increased numbers of intraperitoneal neutrophils (as well as intraperitoneal levels of TNF) in the severe CLP model, but not during infection with *S. typhimurium*. These results raise the possibility that mast cell-derived TNF can have deleterious effects in these two models via distinct mechanisms of action.

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