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Role of mast cells in endomicrobial sepsis revisited: Mast cell-deficient mice show normal immunological protection

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eLife Assessment

This study presents **important** evidence showing that the high susceptibility to sepsis of Kit-mutant mice is not due to mast cell deficiency. The revised manuscript, with **solid** data, focuses on refuting the notion that mast cells play a major role in sepsis. This paper would be of interest to researchers in mast cell biology and mucosal immunology.

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Abstract

Kit-mutant mice are highly susceptible to polymicrobial sepsis elicited by cecal ligation and puncture (CLP). This vulnerability has been attributed to the mast cell deficiency of *Kit* mutants, suggesting key roles of mast cells in antibacterial defense. We show that mice lacking mast cells but wild-type for *Kit* are as resistant to sepsis as mast cell-proficient mice, excluding mast cells as protective factor. Induction of sepsis by direct injection of intestinal microbiota instead of surgical gut perforation revealed comparable protective responses of *Kit*-deficient and *Kit* wild-type mice, indicating intact antibacterial immunity in the absence of Kit. Notably, cecal contents of *Kit*-mutant mice contained 1000-fold greater *Escherichia coli* colony-forming units compared to wild-type mice. Consistently, 16S rRNA gene sequencing revealed a broad compositional shift characterized by enrichment of Enterobacteriaceae and other taxa associated with inflammatory intestinal states, in keeping with intestinal dysmotility in *Kit*-mutant mice. Thus, upon intestinal puncture, overrepresentation of pathogenic bacteria led to a more severe infection of *Kit* mutants compared to wild-type controls. Hence, the susceptibility of *Kit*-mutant mice to sepsis is caused by enteral dysbiosis, not mast cell deficiency. These findings highlight the importance of considering genotype-dependent effects on endogenous microbiota composition in CLP models. Collectively, our results show that immunological resistance to sepsis is independent of mast cells.

Introduction

30 years ago, two hallmark papers studying acute peritoneal infections reported evidence for protective functions of mast cells in anti-bacterial defense[1,2]. These experiments were performed in mice carrying inactivating mutations in the gene encoding the receptor tyrosine kinase Kit ($Kit^{W/Wv}$ mice) which, at the time, were the widely used model of mast cell deficiency owing to the

dependency of mast cell development on Kit expression[3]. Both reports showed that *Kit*$^{W/Wv}$ mice were highly susceptible to peritoneal infections compared with *Kit* wild-type mice. Based on mast cell reconstitution and investigation of associated cytokines, the central conclusion was that the absence of mast cells in *Kit*$^{W/Wv}$ mice, and in particular the absence of mast cell-derived tumor necrosis factor alfa (TNFα), was responsible for the observed immunodeficiency[1,2]. These papers were very influential, and mast cells have since been viewed as key effectors in bacterial defense[4].

At the time, it was well established that *Kit* mutations affect many cell lineages and tissues in developing and adult mice beyond the defect in mast cells, and hence it remained ambiguous whether or not a phenotype observed in *Kit*-mutant mice was indeed due to the absence of mast cells. With the advent of *Kit* mutation-independent mouse models of mast cell deficiency[5–8], which are by comparison to *Kit* mutants highly specific experimental models[9–13], mast cell functions can be probed more conclusively in vivo. It has since become possible to independently confirm, or disprove, what had been concluded earlier on mast cell physiology based on work in *Kit* mutants. Around 15 years into this process of re-evaluation of mast cell functions, a long list of suggested functions have not been reproduced when revisited in *Kit*-independent models (for discussions and references, see[9–13]).

Here, we have re-addressed the important question whether mast cells are involved in immunity to acute peritonitis and sepsis. We confirmed the susceptibility of *Kit*-mutant mice (*Kit*$^{W/Wv}$) to sepsis[1] in the cecal ligation and puncture (CLP) model[14]. However, comparison of *Kit* wild-type mice with (*Cpa3*$^{+/+}$) or without (*Cpa3*$^{Cre/+}$) mast cells[6] showed that the outcome of such infections was independent of mast cells. Furthermore, *Kit*$^{W/Wv}$ mice exhibited the same susceptibility as *Kit*$^{+/+}$ mice upon direct injection of intestinal bacteria, pointing at a defect in *Kit*$^{W/Wv}$ mice related to the intestinal injury, or the release of endogenous flora from the cecum in the CLP model. Finally, we identified markedly higher *Escherichia coli* (*E. coli*) colony-forming units in the cecum of *Kit*$^{W/Wv}$ mice compared to *Kit*$^{+/+}$ mice. At greater resolution, 16S rRNA gene sequencing revealed a pronounced dysbiosis characterized by enrichment of inflammation-associated bacterial taxa in *Kit*$^{W/Wv}$ mice compared to wild-type controls. This dysbiosis is unrelated to mast cell deficiency because mice with (*Cpa3*$^{+/+}$) or without (*Cpa3*$^{Cre/+}$) mast cells had comparable microbiota based on 16S rRNA sequencing. The altered microbial composition in *Kit*$^{W/Wv}$ mice, explains their more severe infection challenge during CLP. Consistent with a microbiota-driven effect, co-housing neutralized the susceptibility of *Kit*-mutant mice to CLP-induced sepsis. Our data suggest that the dysbiosis of *Kit*$^{W/Wv}$ mutants has been misinterpreted as an immunodeficiency. Collectively, we provide evidence that mast cells are dispensable for protection against polymicrobial sepsis and that susceptibility of *Kit*-mutant mice to CLP is driven by elevated microbiota pathogenicity.

Results

Mast cells are dispensable for survival of cecal ligation and puncture-induced polymicrobial sepsis

We subjected mast cell-deficient *Cpa3*$^{Cre/+}$ mice[6] on the C57BL/6 background (*Cpa3*$^{Cre/+}$), and their wild-type littermates (*Cpa3*$^{+/+}$) to the surgical model of cecal ligation and puncture[14]. Under mild sepsis conditions, induced by a 50% ligation of the cecum (tying at the middle between the base and the pole of the cecum) and a single puncture of the cecum with a 25-gauge (25 G) needle, all wild-type mice (*Cpa3*$^{+/+}$) survived (Fig. 1A). Interestingly, all mast cell-deficient *Cpa3*$^{Cre/+}$ mice also survived (Fig. 1A). To explore whether a role for mast cell would become evident under more severe conditions we induced CLP using a 22 G needle (Fig. 1B). The survival (Methods) within two weeks after surgical puncture dropped to about half of the animals for both the *Cpa3*$^{+/+}$ and *Cpa3*$^{Cre/+}$ mice (Fig. 1B). We analyzed large cohorts of about n = 50 for each genotype, and found no difference between both groups (P = 0.23), indicating that the mortality in cecal ligation and puncture sepsis was independent of mast cells. These results were surprising given that previous publications using *Kit*-mutant, mast cell-deficient mice concluded that there was a fundamental requirement for mast cells in sepsis survival[1,2]. As a control, we therefore included

Kit$^{W/Wv}$ mice in our studies. Even under mild sepsis conditions, which all *Kit* wild-type (*Cpa3*$^{+/+}$ or *Cpa3*$^{Cre/+}$) mice survived, 75% of *Kit*$^{W/Wv}$ mice died (Fig. 1A), and in severe sepsis all *Kit*$^{W/Wv}$ mice succumbed the treatment within two days (Fig. 1B). *Kit*$^{W/Wv}$ mice are F1 offspring from WB-*Kit*$^{W/+}$ and B6-*Kit*$^{Wv/+}$ parents, hence they have a genetic F1 hybrid (WBB6F1) background[15] (we refer here to WBB6F1-*Kit*$^{W/Wv}$ mice as *Kit*$^{W/Wv}$ mice). To exclude genetic background differences as a cause of the higher susceptibility of *Kit*$^{W/Wv}$ mice compared to *Cpa3*$^{Cre/+}$ mice, we repeated the CLP experiments using all mice on the WBB6F1 background (WBB6F1-*Cpa3*$^{+/+}$, WBB6F1-*Cpa3*$^{Cre/+}$ and *Kit*$^{W/Wv}$). On this background, mast cell-proficient WBB6F1-*Cpa3*$^{+/+}$ and mast cell-deficient WBB6F1-*Cpa3*$^{Cre/+}$ mice were both even more resistant to CLP than on the B6 background (Fig. 1C), which is consistent with an earlier report[16], and hybrid vigor. In total, 83% of WBB6F1-*Cpa3*$^{+/+}$ and 74% of WBB6F1-*Cpa3*$^{Cre/+}$ mice survived (P = 0.24), while again all *Kit*$^{W/Wv}$ mice succumbed to sepsis. These experiments demonstrate that mast cells are not involved in sepsis resistance, yet *Kit* mutants are highly susceptible.

Because absence of mast cells in *Kit*$^{W/Wv}$ mice has been implicated in impaired cytokine responses in sepsis, notably in TNFα[1,2], we next analyzed the inflammatory cytokine response. Serum levels of TNFα, IL-6, and MCP-1 were measured at three different time points after CLP (Fig. 1D-F). Mast cell-proficient *Cpa3*$^{+/+}$ and mast cell-deficient *Cpa3*$^{Cre/+}$ mice showed very similar time course and amounts of cytokine production. The serum levels for each of the three cytokines was maximal after 8 h, and declined until 24 h without any significant further change at 48 h after cecal ligation and puncture. In contrast, *Kit*$^{W/Wv}$ mice started at 8 h with cytokine levels similar to *Cpa3*$^{+/+}$ and *Cpa3*$^{Cre/+}$ mice, but mounted an almost 10-fold higher TNFα, IL-6 and MCP-1 response at 24 h after CLP (Fig. 1D-F). Sham-treated animals (dashed lines) had transiently increased levels of cytokines at 8 h, in response to the anesthesia and laparotomy (Fig. 1D-F). In Fig. 1G-I we plotted the 24 h serum levels of individual mice, comparing *Cpa3*$^{+/+}$, *Cpa3*$^{Cre/+}$ and *Kit*$^{W/Wv}$ mice. Median cytokine levels were similar in *Cpa3*$^{+/+}$ and *Cpa3*$^{Cre/+}$ mice but elevated in *Kit*$^{W/Wv}$ mice. Comparable cytokine responses in *Cpa3*$^{+/+}$ and *Cpa3*$^{Cre/+}$ mice are consistent with their similar survival rates. Clearly, and in contrast to earlier reports[1,2], the TNFα response was independent of the presence of mast cells, excluding these cells as the main source for TNFα in experimental peritoneal sepsis. Elevate cytokine responses were associated with the *Kit* mutation. Rather than protection, the strongly elevated inflammatory cytokine levels 24 h after cecal ligation and puncture in most *Kit*$^{W/Wv}$, and in some *Cpa3*$^{+/+}$ and *Cpa3*$^{Cre/+}$ mice may reflect an exaggerated immune response before succumbing to sepsis.

Collectively, these experiments demonstrate that mast cells do not play a role in protecting against enteric bacterial sepsis in the CLP model. We confirmed the severely impaired survival of *Kit*$^{W/Wv}$ mice, and found that this was unrelated to deficiencies in production of TNFα, IL-6, or MCP-1.

Mast cell-deficient *Kit*$^{W/Wv}$ mice resist intraperitoneal injection with cecal bacteria

Kit is essential for the development of interstitial cells of Cajal and for intestinal pacemaker activity, and hence autonomous gut motility is largely abrogated in *Kit* mutants[17,18]. Given this dysfunction of the intestinal physiology in *Kit*$^{W/Wv}$ mice, we considered the possibility that the surgical CLP procedure, involving tissue ligation and perforation, could contribute to the sensitivity of *Kit*$^{W/Wv}$ mice to sepsis. We therefore induced sepsis without injury of the colon by injection of a cecal bacterial slurry containing a defined amount of enteral bacteria. This is a reproducible, bacteria dose-controlled peritoneal infection model[19]. Donors for enteral slurry were C57BL/6 mice.

More than 90% of all three genotypes (*Kit*$^{W/Wv}$, *Cpa3*$^{+/+}$ and *Cpa3*$^{Cre/+}$ mice) survived injection of low dose (3 x 10^8) bacteria (Fig. 2A). Survival was reduced to less than 50% during the observation period of 10 days at an approximately 5-fold higher dose (14 x 10^8 bacteria) (Fig. 2B). For both doses, the outcome for *Kit*$^{W/Wv}$ mice was statistically comparable to that of wild-type control mice (Fig. 2A, B), ruling out immunodeficiency to explain the sensitivity of *Kit*$^{W/Wv}$

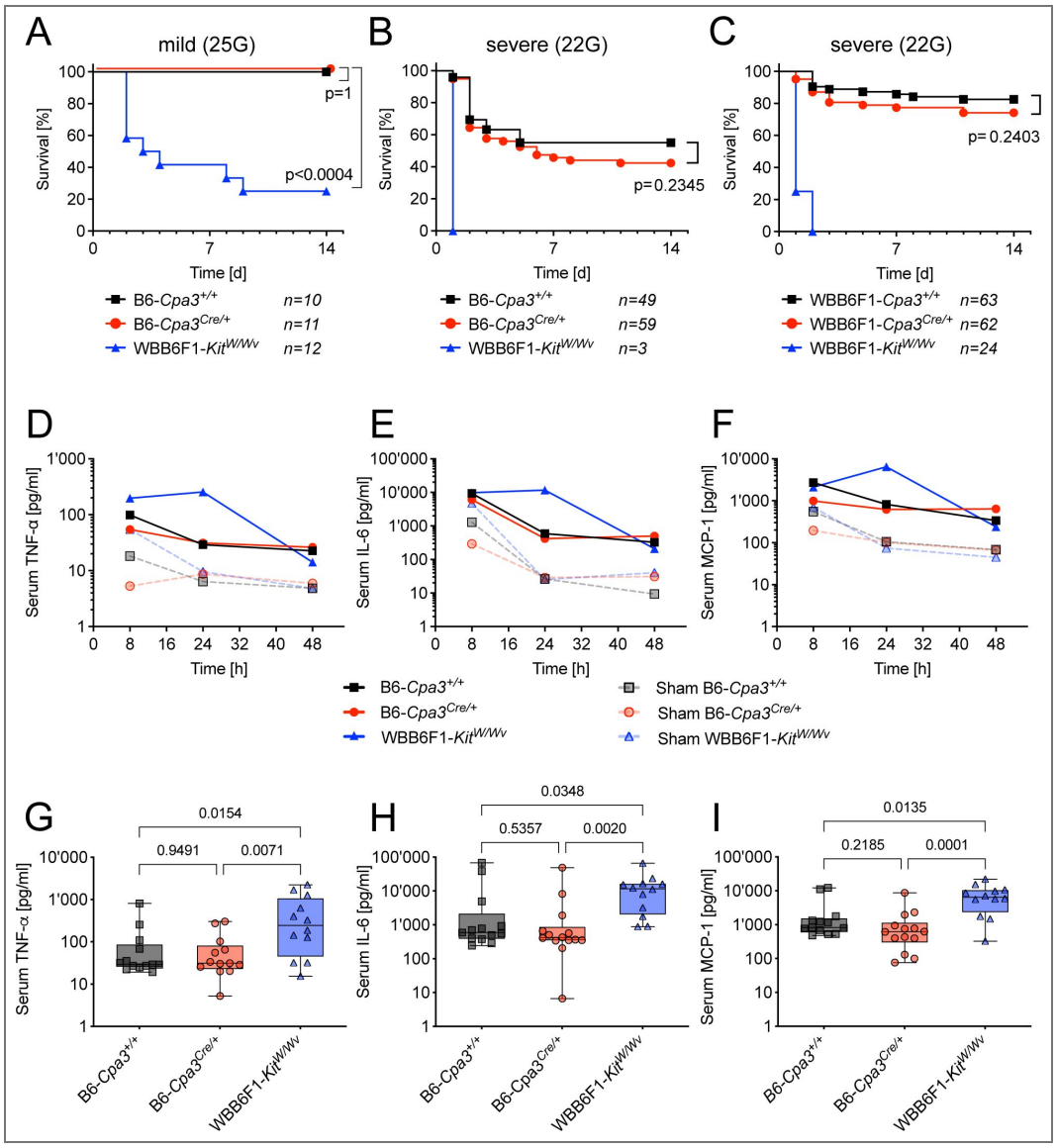

Figure 1. *Kit*$^{W/Wv}$ mutant mice, but not mast cell-deficient *Cpa3*$^{Cre/+}$ mice, are susceptible for cecal ligation and puncture sepsis.

(A). Survival of B6-*Cpa3*$^{+/+}$ (100% of n = 10), B6-*Cpa3*$^{Cre/+}$ (100% of n = 11), and WBB6F1-*Kit*$^{W/Wv}$ (25% of n = 12) mice to mild (25 G needle) cecal ligation and puncture. WBB6F1-*Kit*$^{W/Wv}$ mice vs. B6-*Cpa3*$^{+/+}$ ($P < 0.0004$). (B). Survival of B6-*Cpa3*$^{+/+}$ (55% of n = 49), B6-*Cpa3*$^{Cre/+}$ (42% of n = 59), and WBB6F1-*Kit*$^{W/Wv}$ (0% of n = 3) mice under severe (22 G needle) cecal ligation and puncture. B6-*Cpa3*$^{+/+}$ vs. B6-*Cpa3*$^{Cre/+}$ $P = 0.2345$. WBB6F1-*Kit*$^{W/Wv}$ vs. B6-*Cpa3*$^{+/+}$ $P < 0.0001$. (C). Survival of WBB6F1-*Cpa3*$^{+/+}$ (82.5%, n = 63), WBB6F1-*Cpa3*$^{Cre/+}$ (74.2%, n = 62), and WBB6F1-*Kit*$^{W/Wv}$ (0%, n = 24) mice to severe (22 G needle) cecal ligation and puncture. P values for curve comparisons in A-C were calculated using the Mantel-Cox Log-rank test. (D-F). Kinetics (in hours) of median serum concentrations for TNFα (D), IL-6 (E), and MCP-1 (F) in B6-*Cpa3*$^{+/+}$, B6-*Cpa3*$^{Cre/+}$, and WBB6F1-*Kit*$^{W/Wv}$ mice after severe (22-G needle) cecal ligation and puncture (solid lines), or after sham operations (same procedure but without cecal ligation and puncture) (dashed lines). (G-I). From the kinetic shown in D-F, box plots were drawn for the 24-h time point indicating serum concentrations for TNFα, IL-6, and MCP-1 in B6-*Cpa3*$^{+/+}$, B6-*Cpa3*$^{Cre/+}$ and WBB6F1-*Kit*$^{W/Wv}$ mice. Each dot represents an individual mouse. The boxes extend from the 25th to 75th percentiles and the median is indicated. Whiskers range from minimum to maximum. P values were calculated on log-transformed values by one-way ANOVA with Tukey's correction for multiple comparisons.

mice in cecal ligation and puncture. Mast cell-deficient *Cpa3*$^{Cre/+}$ mice were no more susceptible than their mast cell bearing littermates (Fig. 2A, B), confirming the irrelevance of mast cells in this bacterial injection model.

In order to measure early-stage inflammatory cytokine release, low dose cecal slurry infection was repeated, and mice were analyzed one or two hours later. Compared to NaCl control injected animals, TNFα, IL-6, MCP-1 and IL-10 were elevated in the peritoneal lavage already after one hour, and the cytokine concentrations further increased at two hours after bacteria injection, in particular for IL-6 and MCP-1 (Fig. 2C-F). We could not detect increases in IFN-γ or IL12p70 (data not shown). Statistical comparison of the cytokine responses in *Cpa3*$^{+/+}$, *Cpa3*$^{Cre/+}$ and *Kit*$^{W/Wv}$ mice revealed a significant effect of time, but no significant effects of genotype or genotype *x* time interaction for the cytokine responses, thus excluding mast cells as a major source of inflammatory cytokines, and indicating that this cytokine response is independent of Kit. Since mast cells have been implicated in the recruitment of innate immune cells, we measured neutrophils and macrophages in the peritoneal lavage fluid by flow cytometry one and two hours after bacterial injection (Fig. 2G+H). Neutrophils which are normally absent, or present only at very low numbers in the peritoneal cavity of naïve mice, were rapidly infiltrating the peritoneal cavity of infected animals (Fig. 2G). Of note, we counted very similar numbers of neutrophils (Gr1^{+} CD11b^{+}) in mast cell-proficient *Cpa3*$^{+/+}$, and mast cell-deficient *Cpa3*$^{Cre/+}$ mice. Neutrophil recruitment was slightly reduced in *Kit*$^{W/Wv}$ mice which could be related to their global Kit-dependent neutropenia[20,21]. Numbers of macrophages (F4/80^{+} CD11b^{+}) declined[22] within the first hour of infection in *Cpa3*$^{+/+}$, *Cpa3*$^{Cre/+}$ and *Kit*$^{W/Wv}$ mice (Fig. 2H). As for the cytokine responses, no significant effect of genotype was observed for the neutrophil recruitment or macrophage decline. The significant main effect was time.

Taken together, in contrast to the cecal ligation and puncture sepsis results, *Kit*$^{W/Wv}$ mice show normal survival, cytokine response and neutrophil recruitment after bacterial slurry injection. This implies that the susceptibility of *Kit*$^{W/Wv}$ mice to cecal ligation and puncture sepsis does not reflect defects in immunity, and that immunity is again unaffected by the absence of mast cells. Moreover, our time course experiments show that, within the first hours of peritoneal bacterial infection, mast cells are not major producers of inflammatory cytokines.

Kit$^{W/Wv}$ mice harbor intestinal microflora with increased pathogenic potential

In the CLP model, sepsis is driven by the mouse's own intestinal flora. The selective susceptibility of *Kit*$^{W/Wv}$ mice, but not *Cpa3*$^{Cre/+}$ mice, in this model raises the question whether impaired intestinal physiology, i.e. hypomotility of gut peristalsis and impaired gastrointestinal transit observed in *Kit*-mutants[18,23], may influence the composition and pathogenicity of the intestinal microflora. To directly compare the pathogenicity, we isolated enteral bacteria from either *Kit*$^{+/+}$ or *Kit*$^{W/Wv}$ mice (donor flora) and injected them into recipient cohorts of *Cpa3*$^{+/+}$ (Fig. 3A), *Cpa3*$^{Cre/+}$ (Fig. 3B), or *Kit*$^{W/Wv}$ (Fig. 3C) mice (in this experiment all donor and recipient animals were on the WBB6F1 background). Cecal slurries with five different amounts of bacteria (2, 5, 10, 20, 40 x 10^{8}) were applied. This titration experiment revealed a clear correlation between bacterial load and mortality. Of note, we observed a marked leftward shift in the dose-response curves towards lower bacteria concentrations when comparing injections with cecal slurries from *Kit*$^{W/Wv}$ and *Kit*$^{+/+}$ donors (Fig. 3A-C). Collectively, these data indicate a higher pathogenicity of the microflora harbored in *Kit*-mutants compared to *Kit* wild-type mice.

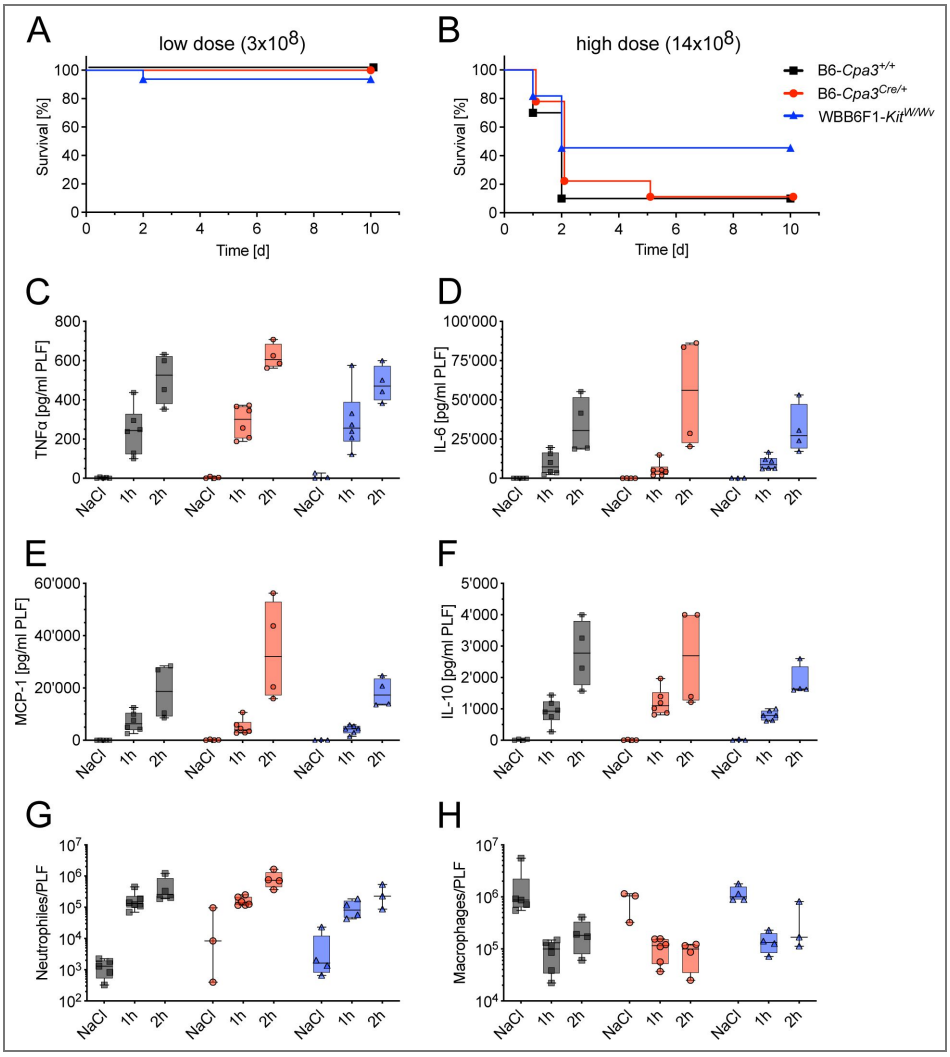

Figure 2. *Kit*$^{W/Wv}$ mice are as resistant to injection of cecal slurry as *Kit*$^{+/+}$ mice.

(A, B). Survival of B6-*Cpa3*$^{+/+}$, B6-*Cpa3*$^{Cre/+}$, and WBB6F1-*Kit*$^{W/Wv}$ mice following injection of low dose (3 x 10^8) bacteria (A), or high dose (14 x 10^8) bacteria (B) from intestines of normal C57BL/6 mice. For both doses, the outcome for *Kit*$^{W/Wv}$ mice was statistically comparable to that of *Kit* wild-type mice, and mast cell deficiency (B6-*Cpa3*$^{+/+}$ versus B6-*Cpa3*$^{Cre/+}$) also played no role in survival. Low dose survival: +/+ 100% of n = 12; *Cre*/+ 100% of n = 16; *W/Wv* 94% of n = 16 and p (+/+ vs *W/Wv*) = 0.3865; high dose survival: +/+ 10% of n = 10; *Cre*/+ 11% of n = 9; *W/Wv* 45% of n = 11 and p (+/+ vs *W/Wv*) = 0.1110. P values of the survival curve comparisons were calculated using the Mantel-Cox Log-rank test. (C-F). Concentration and kinetic of inflammatory cytokine responses in peritoneal lavage fluid from B6-*Cpa3*$^{+/+}$, B6-*Cpa3*$^{Cre/+}$ and WBB6F1-*Kit*$^{W/Wv}$ following saline (NaCl) control injection, or one or two hours after low dose bacterial challenge (3-6 x 10^8 bacteria). Boxes indicate the 25th to 75th percentiles, center lines the median, whiskers the minimum to maximum values, and each dot represents an individual mouse. Statistical analyses were performed on log(Y+1)-transformed values. Ordinary two-way ANOVA with genotype and time as factors revealed no significant effects of genotype or time *x* genotype interaction for any of the cytokines, only significant effects of time were observed (F and P values in Table S1). Sample sizes were n = 6 per genotype at 1 h, n = 4 per genotype at 2 h, and for the NaCl group: n = 5 (+/+), n = 4 (*Cre*/+), and n = 3 (*W/Wv*), except for IL-10 where +/+ mice were n = 4. (G-H). Absolute numbers of (G) neutrophils (Gr1$^+$ CD11b$^+$) and (H) macrophages (Gr1$^-$ CD11b$^+$ F4/80$^+$) in the peritoneal cavity of mice treated as in (C-F). Statistical analyses were performed on log(Y)-transformed values using ordinary two-way ANOVA with genotype and time as factors. Significant effects of time, but no effects of genotype or time *x* genotype interaction, were observed. (F and P values in Table S1). Sample sizes were for NaCl: n = 5 (+/+), n = 3 (*Cre*/+), and n = 4 (*W/Wv*); at 1h: n = 6 (+/+), n = 6 (*Cre*/+), and n = 4 (*W/Wv*); and at 2h n = 4 (+/+), n = 4 (*Cre*/+), and n = 3 (*W/Wv*).

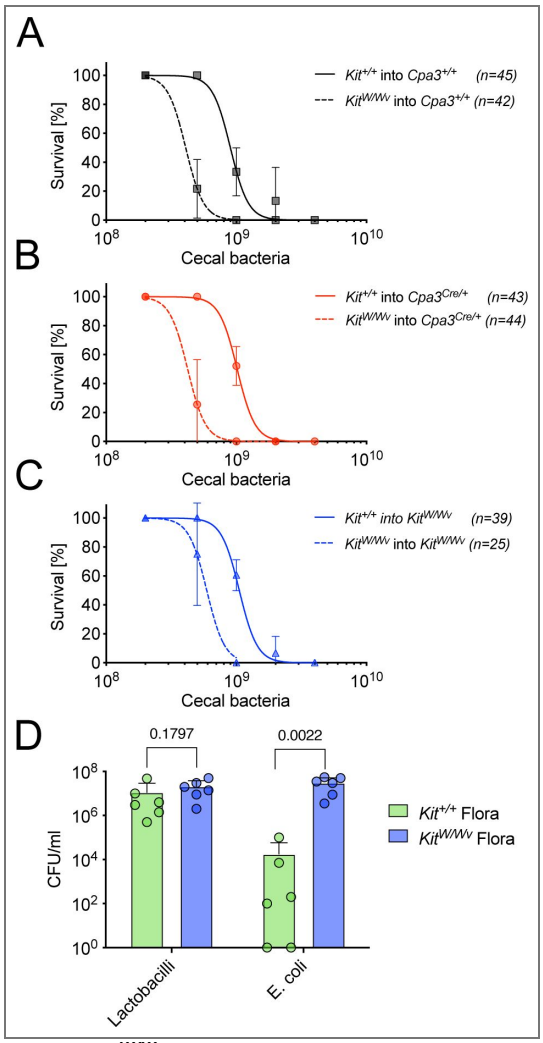

Figure 3. *Kit*$^{W/Wv}$ mice harbor more pathogenic enteral bacteria than *Kit*$^{+/+}$ mice. (A-C). Survival of *Cpa3*$^{+/+}$ (A), *Cpa3*$^{Cre/+}$ (B), and *Kit*$^{W/Wv}$ (C) mice following injection of increasing doses of bacteria (2, 5, 10, 20 or 40 x 10^8) isolated from the cecum of *Kit*$^{+/+}$ or *Kit*$^{W/Wv}$ donor mice. All animals in this experiment were on the WBB6F1 strain background. Group sizes (n) are given in the figure. (D). Bacterial colony forming units were determined for cecal slurries of *Kit*$^{+/+}$ (n = 6) and *Kit*$^{W/Wv}$ mice (n = 6) which included all cecal slurries used in (A-C). Bars graphs show the mean + SD, each dot represents counts from one individual mouse. P values were calculated with the Mann-Whitney test.

To investigate whether the increased pathogenicity of *Kit*$^{W/Wv}$ microbiota is due to an altered bacterial composition of the cecal contents, we first performed a basic microbiological examination and determined bacterial colony forming units (CFU) of the intestinal contents of *Kit*$^{+/+}$ and *Kit*$^{W/Wv}$ mice, including the specimens used for the i.p. infection experiments shown in Fig. 3A-C. To this end, we prepared serial dilutions of the cecal slurries, cultured these on blood agar and McConkey agar plates, and counted bacterial colonies. While Lactobacilli colonies were obtained at similar frequencies from isolates of both mouse strains, CFU counts for *Escherichia coli* (*E. coli*) were more that 1000 times higher in *Kit*$^{W/Wv}$ than in *Kit*$^{+/+}$ isolates (Fig. 3D). *Staphylococcus sp.* (most likely *Staph. Xylosus*) was sporadically found at low level in *Kit*$^{+/+}$ slurries (not shown). Hence, *Kit*$^{W/Wv}$ microbiota contains high levels of *E. coli*, which may underlie the observed pathogenicity.

Kit deficiency, but not mast cell deficiency, is associated with intestinal dysbiosis

For a more comprehensive analysis of the intestinal microbiome, we performed 16S rRNA sequencing. The V6 region of the bacterial 16S rRNA gene was amplified and sequenced from cecal samples collected from $Kit^{W/Wv}$ mice and $Kit^{+/+}$ littermate controls (Fig. 4A+B), as well as from $Cpa3^{Cre/+}$ mice and $Cpa3^{+/+}$ littermate controls (Fig. 4C+D). Amplicon reads were taxonomically classified, and relative abundances were calculated at the order and family levels.

At the bacterial order level mean relative abundances revealed similar microbial community structures that were dominated by Clostridiales and Bacteroidales, irrespective of the genotype of the mice (Fig. 4A+C). Nevertheless, host genotype-associated shifts were observed for specific taxa in $Kit^{W/Wv}$ mice (Fig. 4A outer circle) exhibiting increased relative abundances of Verrucomicrobiales, Enterobacteriales, and Erysipelotrichales, accompanied by a reciprocal decrease in Bacteroidales relative to $Kit^{+/+}$ controls (inner circle). Lactobacillales and Bifidobacteriales were present at comparable abundances in both genotypes.

To assess host genotype-associated differences in microbiome composition at higher taxonomic resolution, the bacterial family-level read count matrix was analyzed using the statistical package ALDeX2. For visualization, relative abundances were displayed as grouped bar charts depicting individual mice with separate bars (Fig. 4B+D).

Differential abundance analysis found the bacterial families Peptostreptococcaceae, Verrucomicrobiaceae, Enterobacteriaceae, Coriobacteriaceae, and Erysipelotrichaceae enriched in $Kit^{W/Wv}$ mice compared to $Kit^{+/+}$ controls, exhibiting moderate-to-large effect sizes (Table S2). In particular, Enterobacteriaceae, Peptostreptococcaceae, and Verrucomicrobiaceae displayed some of the largest positive effect sizes observed in the dataset. Although, these differences did not remain statistically significant after FDR correction, the large differential abundances and effect sizes suggest a biologically relevant restructuring of the microbial community towards facultative anaerobic and potentially pro-inflammatory bacterial families in $Kit^{W/Wv}$ mice. Moreover, the marked increase in Enterobacteriaceae (diff_abundance = 8.24) in $Kit^{W/Wv}$ mice, together with the largely unaltered abundance of Lactobacillaceae (effect = -0.54, FDR = 0.939), was consistent with the CFU counts obtained from the cecal slurry titration assay (Fig. 3D). In contrast to these increases, Bacteroidaceae were significantly reduced in $Kit^{W/Wv}$ mice compared to $Kit^{+/+}$ controls (effect = −2.40, FDR = 0.018) (Table S2).

Interestingly, in contrast to the pronounced microbiome alterations observed in $Kit^{W/Wv}$ mice, the microbiome composition of $Cpa3^{Cre/+}$ mice was largely comparable to that of $Cpa3^{+/+}$ littermate controls (Fig. 4C+D). ALDEx2 analysis revealed only small effect sizes (effect < 0.7), uniformly high FDR values (FDR > 0.98), and no coherent directional pattern across phylogenetically related bacterial families (Fig. 4D and Table S3).

Collectively, these findings indicate that Kit deficiency, but not mast cell deficiency, is the major determinant of the microbiome alteration observed in $Kit^{W/Wv}$ mice, and that the increased pathogenicity of their intestinal flora is associated with a dysbiotic shift toward bacterial taxa associated with intestinal inflammation.

Cohousing transfers sepsis resistance to $Kit^{W/Wv}$ mice

To obtain further evidence that pathogenic microbiota in $Kit^{W/Wv}$ mice is responsible for the increased CLP-susceptibility of this strain independent of mast cells, we performed co-housing experiments. $Kit^{W/Wv}$ and their wild-type $Kit^{+/+}$ littermates are F1 offspring from WB-$Kit^{W/+}$ and C57BL/6-$Kit^{Wv/+}$ parents. The phenotypically different $Kit^{W/Wv}$ (white) and $Kit^{+/+}$ (black) mice were normally separated at weaning, which may result in genotype-specific drifts of the enteric microbiota. To prevent such drift, we co-housed $Kit^{W/Wv}$ and $Kit^{+/+}$ mice, and repeated cecal ligation and puncture experiments. In contrast to the previous CLP experiments with non-co-housed mice in which severe (22 G needle) conditions were lethal for all $Kit^{W/Wv}$ mice (Fig. 1B),

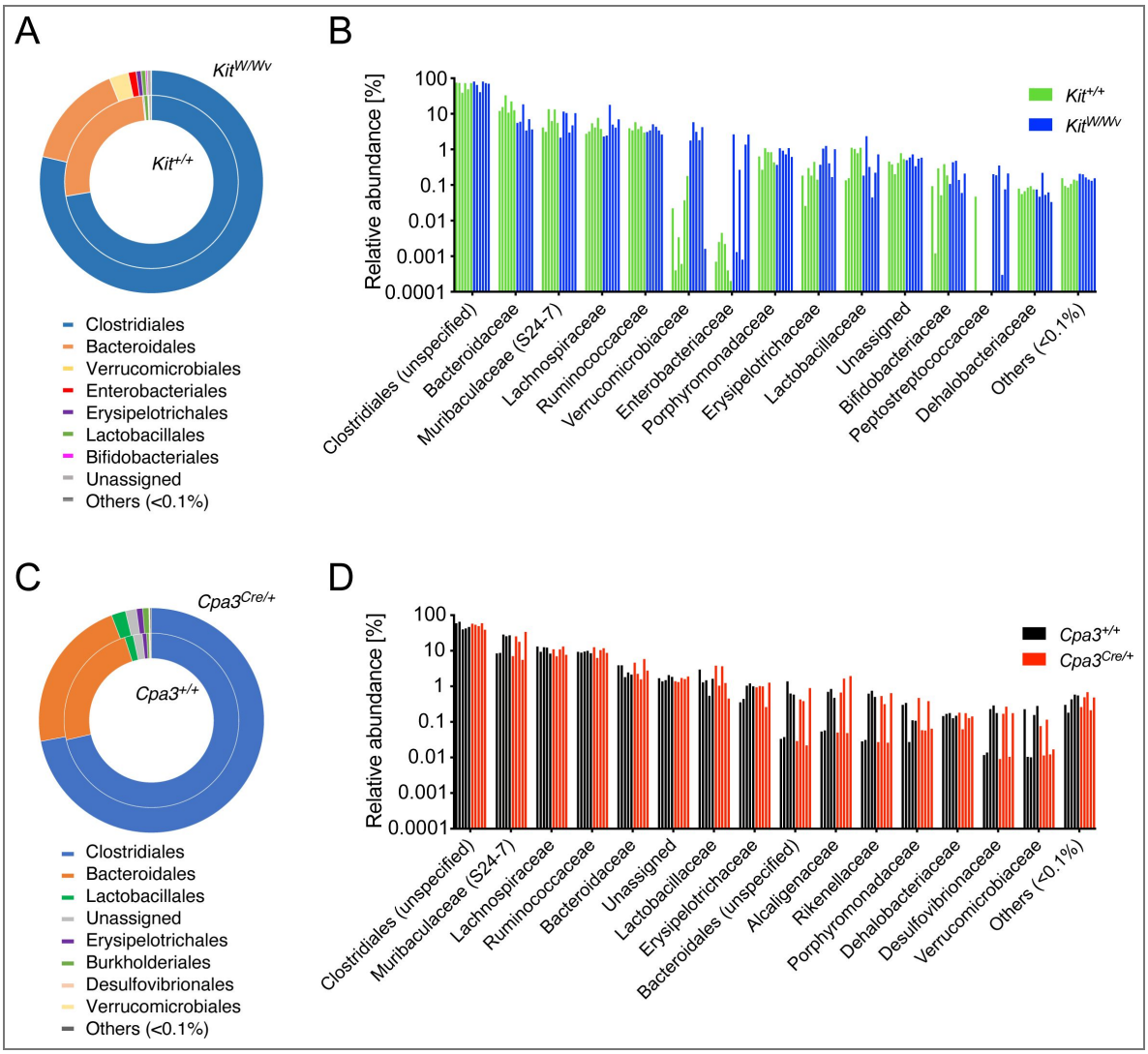

Figure 4. Cecal microbiota composition in *Kit*$^{W/Wv}$ and *Cpa3*$^{Cre/+}$ mice compared with their respective littermates.

(A) Community composition of bacterial orders in cecal samples from *Kit*$^{W/Wv}$ (outer circle) and *Kit*$^{+/+}$ (inner circle) mice shown as mean relative abundances (n = 6 per group). (B) Relative abundance of bacterial families in individual mice shown as grouped bar plots organized by family. Bars represent data of individual *Kit*$^{+/+}$ (green) and *Kit*$^{W/Wv}$ (blue) animals. Statistical analysis was performed using ALDeX2. (C) Community composition of bacterial orders in cecal samples from *Cpa3*$^{Cre/+}$ (outer circle) and *Cpa3*$^{+/+}$ (inner circle) mice shown as mean relative abundances (n = 5 per group). (D) Relative abundance of bacterial families in individual mice shown as grouped bar plots organized by family. Bars represent data of individual *Cpa3*$^{+/+}$ (black) and *Cpa3*$^{Cre/+}$ (red) animals. Statistical analysis was performed using ALDeX2. Taxa with a relative abundance >0.1% in at least one sample within each panel are shown individually; all other taxa were summed up in "Others".

after co-housing there were no differences in survival (P = 0.3809) comparing *Kit*$^{W/Wv}$ (74% of n = 38) and *Kit*$^{+/+}$ (65% of n = 25) mice (Fig. 5). These experiments demonstrate that co-housing transfers CLP-resistance from *Kit*$^{+/+}$ to *Kit*$^{W/Wv}$ mice.

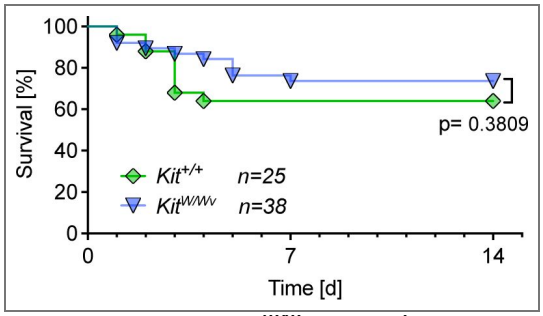

Figure 5. Co-housing of *Kit*$^{W/Wv}$ with *Kit*$^{+/+}$ mice normalizes the susceptibility of *Kit*$^{W/Wv}$ mice to cecal ligation and puncture. *Kit*$^{+/+}$ and *Kit*$^{W/Wv}$ mice (WBB6F1 background) were housed together from weaning onwards until cecal ligation and puncture (22 G needle). This constant co-housing, increased the survival rate of *Kit*$^{W/Wv}$ mice (n=38) and equalized it (p=0.3809) to the probability of survival of *Kit*$^{+/+}$ mice (n=25). P values were calculated using the Mantel-Cox Log-rank test.

Discussion

Kit$^{W/Wv}$ mice exhibit increased mortality compared to *Kit*$^{+/+}$ control mice[1] in the clinically relevant cecal ligation and puncture (CLP) model of polymicrobial sepsis[14]. This phenotype has been attributed to the absence of mast cells, and broadly been interpreted as evidence for a non-redundant role of mast cells in antibacterial defense during systemic infection. In the present study, we re-examined CLP sepsis experiments using *Cpa3*$^{Cre/+}$ mice, a genetic model that separates mast cell deficiency from other pathophysiology in *Kit* mutants. *Cpa3*$^{Cre/+}$ mice, which lack mast cells but have intact Kit signaling, showed survival rates comparable to wild-type controls in response to CLP. This finding was highly reproducible across experiments performed over eight years by three independent investigators at two institutions, using large experimental cohorts, supporting the robustness of this observation. Clearly, this large dataset refutes a protective role for mast cells in polymicrobial sepsis elicited by cecal ligation and puncture (CLP).

There are conflicting reports regarding the role of mast cells in the cecal ligation and puncture assay, or in related bacterial infection models. Published results range from pro-pathogenic roles of mast cells[24] to enhanced vulnerability of mouse mutants with loss of protease genes (Mcpt4; Mcpt6)[16,25]. These reports are difficult to reconcile with our findings demonstrating unimpaired survival of mast cell-deficient (*Cpa3*$^{Cre/+}$) mice. In a peritonitis model induced by injection of a mouse-virulent strain of *Klebsiella pneumoniae*, *Kit*$^{W/Wv}$ mice were reported to exhibit impaired bacterial clearance, reduced neutrophil recruitment, and increased expression of TNFα in the peritoneal cavity[2]. In our hands, injecting the 'natural mixture' of cecal bacteria for intraperitoneal infections, *Kit*$^{W/Wv}$ mice showed comparable survival, TNFα secretion and neutrophil recruitment to the peritoneal cavity, all of which does not agree with the immunodeficiency that Malaviya et al. described[2]. The reasons for these discrepancies are unclear. Naturally, host-to-host transmission of *Klebsiella pneumoniae* requires close contact and generally occurs through the fecal-oral route[26] rather than experimental intraperitoneal infections. Rather than examining host defense against single bacterial strains, such as *Klebsiella pneumoniae* or *E. coli*, we chose a more natural polymicrobial model, mimicking the release of enteral bacteria following gastrointestinal rupture, and found no evidence for a protective role of mast cells. This conclusion aligns with recent proteome data obtained from primary mouse and human mast cells[27]. By comparison with macrophages, ex vivo isolated mast cells lacked several different classes of pattern recognition receptors which suggests that peritoneal mast cells, at least at steady state, do not possess broad innate pattern recognition capabilities. Specifically, primary human and mouse mast cells do not express detectable amounts of TLRs, and their expression of

MYD88 most likely does not represent a configuration for TLR but rather for IL-1R signaling. Mast cells share expression of the RNA sensor RIG-I and its downstream transcription factor IRF3 with most other immune cells, but do not express other RIG-I-like receptors or their downstream signaling components. Mast cells also do not express C-type lectin receptor components, or inflammasome components[27]. Importantly, TNFα protein, reported to be stored in mast cell secretory granules in vitro[28], was also undetectable in primary mast cells[27]. These data, together with our polymicrobial sepsis experiments, make it likely that mast cells play no role in protection against peritoneal sepsis. Instead, it appears that the principal innate responder cells in peritoneal sepsis are macrophages and neutrophils which are also the main cellular sources of TNFα.

How is it possible that the opposite conclusion, i.e. that mast cells are key in protection from sepsis, had been reached 30 years ago based on experiments in *Kit* mutants? Or, in other words, if their mast cell deficiency is not causal for sepsis susceptibility, what renders *Kit* mutants so susceptible? We also addressed this question. The answer came from focusing in particular on intestinal flora. When we challenged $Kit^{W/Wv}$ mice, which are also in our hands sensitive to CLP sepsis, through intraperitoneal injection of intestinal bacterial suspensions ("cecal slurry") from wild type intestines, $Kit^{W/Wv}$ mice were as resistant as normal mice to the infection. Of note, $Kit^{W/Wv}$ mice exhibited survival kinetics, cytokine responses, and neutrophil recruitment indistinguishable to $Cpa3^{Cre/+}$ mice or $Cpa3^{+/+}$ controls. Interestingly, we next discovered that cecal slurries prepared from $Kit^{W/Wv}$ intestines were more pathogenic and contained substantially higher *E. coli* CFU than those from wild-type controls. In brief, the increased susceptibility of *Kit*-mutant mice to CLP is unrelated to mast cells or other defects in antibacterial immunity, but instead reflects differences in the infectious inoculum released upon intestinal injury.

Consistent with the impaired survival and *E. coli* CFU data, 16S rRNA sequencing of cecal samples revealed an altered microbial ecosystem in $Kit^{W/Wv}$ mice compared to $Kit^{+/+}$ controls. *Kit*-mutant mice exhibited a compositional shift characterized by an enrichment of Enterobacteriaceae, Verrucomicrobiaceae, Erysipelotrichaceae, and Peptostreptococcaceae, along with a reduction in Bacteroidaceae. Members of the Enterobacteriaceae family, including Escherichia coli, are facultatively anaerobic Gram-negative bacteria with a well-established pathogenic potential in sepsis. They are frequent drivers of inflammation following intestinal barrier disruption[29,30]. Likewise, Erysipelotrichaceae and Peptostreptococcaceae have been associated with intestinal inflammation, inflammatory bowel disease, and microbiota instability in both humans and experimental models[29,31]. Notably, the increase of Erysipelotrichaceae observed in $Kit^{W/Wv}$ mice was mainly attributed to the genus *Allobaculum*. Recent studies identified *Allobaculum mucolyticum* as a mucin-degrading bacterium in the intestines of IBD patients, suggesting that an expansion of this genus may reflect altered ecological conditions in the mucosa during inflammatory diseases[32]. A parallel expansion of Peptostreptococcaceae and Enterobacteriaceae, together with a concomitant decrease of Bacteroidaceae, has been reported for example in colitis and IBD patients[33,34]. Collectively, the microbial changes we observed in $Kit^{W/Wv}$ mice resemble dysbiotic patterns reported in chronic intestinal inflammation, experimental colitis, and impaired barrier function. We note that the observed microbiome differences are correlative and do not formally demonstrate which specific bacterial taxa were responsible for the increased pathogenicity observed in the CLP or cecal slurry assays.

Given that $Cpa3^{Cre/+}$ mice relative to $Cpa3^{+/+}$ controls have an unaltered intestinal microbiome, we exclude mast cell deficiency as the causal factor for the underlying dysbiosis in $Kit^{W/Wv}$ mice. *Kit*-mutant mice suffer from a known intestinal motility disorder, resulting from impaired development and maintenance of interstitial cells of Cajal[17,18]. While it was not a focus of this work to directly address the relationship between intestinal motility and microbial composition, the microbiota alterations identified by 16S rRNA gene sequencing are compatible with delayed intestinal transit reshaping the gut microbial ecosystem. This condition may favor the expansion of facultative anaerobic bacteria such as Enterobacteriaceae, and thus contribute to a dysbiotic microbial community associated with intestinal inflammation.

The differences in composition and pathogenicity of *Kit*-mutant and wild-type cecal microbiota we report here can only directly refer to the mice bred and maintained during the experimental period in our mouse facilities. Breeding $Kit^{+/+}$ and $Kit^{W/Wv}$ mice from WB-$Kit^{W/+}$ and B6-$Kit^{Wv/+}$ parents, and separating the offspring at the time of weaning, led to the observed differences in microbiota within the same animal facility. Performing sepsis experiments in mice generated in this manner revealed that lack of Kit can strongly enhance the pathogenicity and, in particular, markedly increase numbers of *E. coli* colony-forming units. We suggest assessing beforehand whether microbiota are, in fact, comparable between wild-type and mutant mice in CLP sepsis experiments. If they are not, as in the case of the *Kit* mutants, the experimental and the control group may release incomparable microbiota from the intestinal puncture. This consideration also applies to experimental and control mice obtained from different breeders or housed separately.

Collectively, our experiments show that mast cells play no role in protection from endomicrobial sepsis, and they also provide an explanation for the apparent immunological susceptibility and mast cell-dependent phenotype of *Kit* mutants in this infection.

Methods

Mice

B6-$Cpa3^{Cre/+}$ mice were obtained after backcrossing the $Cpa3^{Cre}$ allele ($Cpa3^{tm3(icre)Hrr}$)[6] for >20 generations onto C57BL/6J (B6). In all experiments, littermates from the breeding of B6-$Cpa3^{Cre/+}$ with wild-type B6 mice were used. *Kit*-mutant mast-cell-deficient WBB6F1-$Kit^{W/Wv}$ mice and their WBB6F1-$Kit^{+/+}$ littermate controls were obtained by crossing WB-$Kit^{W/+}$ with B6-$Kit^{Wv/+}$ mice (both originally obtained from Japan-SLC, Shizuoka, Japan). Unless otherwise described in the text, WBB6F1-$Kit^{W/Wv}$ mice and their WBB6F1-$Kit^{+/+}$ littermates were separated by coat color at weaning. For direct comparison, WBB6F1-$Cpa3^{Cre/+}$ and WBB6F1-$Cpa3^{+/+}$ littermates were generated in F1 intercrosses of WB-$Kit^{+/+}$ with B6-$Cpa3^{Cre/+}$ mice.

All animals were generated at the mouse breeding facilities of the University Clinics in Ulm or the center for preclinical research at the DKFZ in Heidelberg. All experiments were conducted in accordance to animal care guidelines pertaining to local animal committees (Regierungspräsidium Tübingen or Regierungspräsidium Karlsruhe) and to the institutional guidelines.

Sepsis models

Cecal ligation and puncture (CLP)

The surgical sepsis model was performed according to Rittirsch et al.[35] and Cuenca et al.[36]. In brief, mice were anesthetized (100 mg/kg ketamine and 16 mg/kg xylazine in saline, i.p.), belly shaved and placed onto a sterile-covered warming plate for the duration of the surgery. After disinfection with 70% ethanol, the abdomen of the mice was opened with scissors by a 1-cm mid line cut into the skin, followed by a 1-cm incision of the peritoneum. The cecum was exteriorized and its stool content was gently palpated towards the distal end. The ligation with 4-0 sutures was placed at half the distance (‘50% ligation’) between the distal pole and the base of the cecum. A single puncture was applied to the pole of the cecum (gauge sizes of the used needles are indicated for each experiment). Gentle pressure on the ligated cecum released a tiny drop of the cecal content while the needle was withdrawn to prevent immediate closure of the puncture hole. Afterwards the cecum was reposed and wound closure was performed with surgical clips. Sham control mice underwent the same surgical procedure, however without ligation and puncture of the cecum. Finally, mice received 1 ml saline s.c. for fluid replacement. For the entire observation period after surgery, all mice were closely monitored, and animals showing signs of severe distress or suffering were euthanized. Hence, statements in this manuscript referring to mortality all fell under this animal welfare protocol. For profiling of inflammatory cytokines, mice were sacrificed at the indicated time after surgery and blood for serum preparation was collected by heart puncture.

Cecal slurry injection

For the non-surgical sepsis model, suspensions of cecal bacteria were prepared from donor mice and injected intraperitoneally into the test mice. For preparation of the cecal slurry, four donor mice (B6-*Cpa3*$^{+/+}$ (Fig. 2), WBB6F1-*Kit*$^{+/+}$ or WBB6F1-*Kit*$^{W/Wv}$ (Fig. 3)) were sacrificed and the content of their cecum was pooled and resuspended in 10 ml saline (0.9 M NaCl). The suspension was centrifuged for 1 min at 20 g (200 rpm) in a swing-out bucket and the upper 8 ml were subsequently passed through 100 µm and 40 µm filters. Bacteria counts (rod-shaped) were determined using a Neubauer counting chamber with 0.02 mm chamber depth. Injected bacterial doses are indicated in Figures 2 and 3. Injected mice were closely monitored and moribund mice were sacrificed (see 'Cecal ligation and puncture' paragraph above). For the analysis of cytokine concentrations and myeloid cell infiltrations in the peritoneal cavity, mice were sacrificed at the indicated time after cecal slurry injection and the peritoneal fluid was collected. Therefore, 700-800 µl sterile FACS buffer (PBS with 5% FCS) were injected intraperitoneally, and after a short abdominal massage, the peritoneal cavity was opened by a 5 mm incision to retrieve about 500 µl of the peritoneal exudate suspension with a pipette. A small aliquot was used to determine the concentration of cells and the total amount of cells was calculated from the total injected volume. The peritoneal exudate suspension was then separated into a cellular and a soluble fraction by centrifugation for 5 min with 500 g (2200 rpm). The cell pellet was resuspended in FACS buffer for subsequent flow cytometric analysis. The soluble fraction was centrifuged for another 5 min at 16200 g (13000 rpm) and the supernatant was snap frozen in liquid nitrogen for later cytokine measurement.

CFU determination

CFU of the cecal slurry suspensions used in the sepsis experiment was determined by plating serial dilutions (10^{-3} – 10^{-10} in 0.9 M NaCl) of the slurry stock suspension in replicates on blood agar and McConkey agar. *Lactobacillus* and *Staphylococcus* colonies were counted on blood agar plates, *Escherichia coli* colonies on McConkey agar plates.

Microbiome analysis

Mice were housed in individually ventilated cages under barrier conditions at the central animal facility of the German Cancer Research Center, Heidelberg, and fed standard breeding chow (Kliba 3307). After weaning, littermates were separated and maintained in individual cages for at least nine weeks before sacrifice and cecal content collection. DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen). The V6 region of the bacterial 16S rRNA gene was amplified using indexed primers (see Table S4) under the following conditions: 94 °C for 3 min; 32 cycles of 94 °C for 45 s, 52 °C for 60 s, and 72 °C for 90 s; followed by 72 °C for 10 min. PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and equimolarly pooled for library preparation at the NGS core facility. Paired-end sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Raw reads were quality-trimmed and denoised using DADA2 (R v3.6.3). Resulting amplicon sequence variants were taxonomically classified at phylum, class, order, family, and genus levels using the SILVA database (v132). Sequencing depth ranged from 90k to 850k reads per sample.

Differential abundance analysis at the family level was performed in R (v4.6.0) using ALDEx2 (v1.44.0) on raw count data. Taxa were filtered by prevalence (≥20% of samples) and total abundance (>5 reads across all samples) prior to analysis. ALDEx2 used 128 Monte Carlo Dirichlet instances and centered log-ratio-transformed counts to estimate effect sizes, differential abundance, and Welch's t-test P values with Benjamini–Hochberg FDR correction.

Relative abundances profiles were calculated by normalizing raw read counts to total sequencing depth per sample. In graphical illustrations, low-abundance taxa with a mean relative abundance <0.1% were collapsed into a single "Others (<0.1%)" category.

Flow cytometric assays

Cytometric bead array (CBA)

Cytokine concentrations of serum samples or peritoneal lavages were determined in a bead-based multiplex cytometric assay (Cytometric Bead Array (CBA) Mouse Inflammation Kit, BD Biosciences). Samples (undiluted and 1:20 dilutions) and cytokine standards (10 ng – 5 pg serial dilutions) were incubated with mixtures of fluorescent beads coated with capture antibodies against IL-6, IL-10, INF-γ, MCP-1, TNF-α and IL12p70. PE-labeled detection reagent (i.e. PE-conjugated antibodies specific for the respective mouse cytokines) was added, and, after incubation and wash, samples were measured at a BD FACSCanto^{TM} or BD LSRFortessa^{TM} instrument. Cytokine concentrations were calculated from mean fluorescence values using the BD FCAP Array Software.

Peritoneal exudate cells (PEC)

The cellular fraction of the peritoneal exudate suspensions was analyzed for CD117^{+} mast cells, Gr1^{+} CD11b^{+} granulocytes and Gr1^{-} CD11b^{+} F4/80^{+} macrophages on a BD FACS Canto. Therefore, cells were first incubated with mouse IgG (300 µg/ml, Dianova) for blocking of Fc-receptors and then stained with titrated amounts of fluorescent-labeled antibodies in PBS with 5% FCS. The following antibodies were used: CD117-APC (2B8), CD11b-FITC (M1/70), Gr1-PE (RB6-8C5) all from Pharmingen, and F4/80-APC-A780 (BM8) from eBioscience.

Data availability

The entire data are available from the authors (t.feyerabend@dkfz.de) and will be placed in a public repository in due course.

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Additional files

Supplemental Items

Additional information

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References

1. **Echtenacher B,** Männel DN, Hültner L (1996) Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* **381**:75-77 https://doi.org/10.1038/381075a0 | PubMed
2. **Malaviya R,** Ikeda T, Ross E, Abraham SN (1996) Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α. *Nature* **381**:77-80 https://doi.org/10.1038/381077a0 | PubMed
3. **Kitamura Y,** Go S, Hatanaka K (1978) Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. *Blood* **52**:447-452 https://doi.org/10.1182/blood.v52.2.447.bloodjournal522447 | PubMed
4. **Abraham SN,** St John AL (2010) Mast cell-orchestrated immunity to pathogens. *Nat Rev Immunol* **10**:440-452 https://doi.org/10.1038/nri2782 | PubMed
5. **Dudeck A,** Dudeck J, Scholten J, Petzold A, Surianarayanan S, Köhler A, Peschke K, Vöhringer D, Waskow C, Krieg T, *et al.* (2011) Mast Cells Are Key Promoters of Contact Allergy that Mediate the Adjuvant Effects of Haptens. *Immunity* **34**:973-984 https://doi.org/10.1016/j.immuni.2011.03.028 | PubMed
6. **Feyerabend TB,** Weiser A, Tietz A, Stassen M, Harris N, Kopf M, Radermacher P, Möller P, Benoist C, Mathis D, *et al.* (2011) Cre-Mediated Cell Ablation Contests Mast Cell Contribution in Models of Antibody- and T Cell-Mediated Autoimmunity. *Immunity* **35**:832-844 https://doi.org/10.1016/j.immuni.2011.09.015 | PubMed
7. **Lilla JN,** Chen C.-C, Mukai K, BenBarak MJ, Franco CB, Kalesnikoff J, Yu M, Tsai M, Piliponsky AM, Galli SJ (2011) Reduced mast cell and basophil numbers and function in Cpa3-Cre; Mcl-1fl/fl mice. *Blood* **118**:6930-6938 https://doi.org/10.1182/blood-2011-03-343962 | PubMed
8. **Otsuka A,** Kubo M, Honda T, Egawa G, Nakajima S, Tanizaki H, Kim B, Matsuoka S, Watanabe T, Nakae S, *et al.* (2011) Requirement of Interaction between Mast Cells and Skin Dendritic Cells to Establish Contact Hypersensitivity. *Plos One* **6**:e25538 https://doi.org/10.1371/journal.pone.0025538 | PubMed
9. **Rodewald H.-R,** Feyerabend TB (2012) Widespread Immunological Functions of Mast Cells: Fact or Fiction?. *Immunity* **37**:13-24 https://doi.org/10.1016/j.immuni.2012.07.007 | PubMed
10. **Feyerabend TB,** Gutierrez DA, Rodewald H.-R (2016) Of Mouse Models of Mast Cell Deficiency and Metabolic Syndrome. *Cell Metab* **24**:1-2 https://doi.org/10.1016/j.cmet.2016.06.019 | PubMed
11. **Schubert N,** Lisenko K, Auerbach C, Weitzmann A, Ghouse SM, Muhandes L, Haase C, Häring T, Schulze L, Voehringer D, *et al.* (2018) Unimpaired Responses to Vaccination With Protein Antigen Plus Adjuvant in Mice With Kit-Independent Mast Cell Deficiency. *Front Immunol* **9**:1870 https://doi.org/10.3389/fimmu.2018.01870 | PubMed
12. **Maurer M,** Taube C, Schröder NWJ, Ebmeyer J, Siebenhaar F, Geldmacher A, Schubert N, Roers A (2019) Mast cells drive IgE-mediated disease but might be bystanders in many other inflammatory and neoplastic conditions. *J Allergy Clin Immun* **144**:S19-S30 https://doi.org/10.1016/j.jaci.2019.07.017 | PubMed
13. **Galli SJ,** Gaudenzio N, Tsai M (2020) Mast Cells in Inflammation and Disease: Recent Progress and Ongoing Concerns. *Annu Rev Immunol* **38**:49-77 https://doi.org/10.1146/annurev-immunol-071719-094903 | PubMed
14. **Wichterman KA,** Baue AE, Chaudry IH (1980) Sepsis and septic shock--a review of laboratory models and a proposal. *J Surg Res* **29**:189-201 https://doi.org/10.1016/0022-4804(80)90037-2 | PubMed

15. **Russell ES** (1979) Hereditary anemias of the mouse: a review for geneticists. *Adv. Genet* **20**:357-459 https://doi.org/10.1016/s0065-2660(08)60549-0 | PubMed

16. **Piliponsky AM,** Chen C.-C, Rios EJ, Treuting PM, Lahiri A, Abrink M, Pejler G, Tsai M, Galli SJ (2012) The Chymase Mouse Mast Cell Protease 4 Degrades TNF, Limits Inflammation, and Promotes Survival in a Model of Sepsis. *Am J Pathology* **181**:875-886 https://doi.org/10.1016/j.ajpath.2012.05.013 | PubMed

17. **Maeda H,** Yamagata A, Nishikawa S, Yoshinaga K, Kobayashi S, Nishi K, Nishikawa S.-I (1992) Requirement of c-kit for development of intestinal pacemaker system. *Development* **116**:369-375 https://doi.org/10.1242/dev.116.2.369 | PubMed

18. **Hulzinga JD,** Thuneberg L, Klüppel M, Malysz J, Mikkelsen HB, Bernstein A (1995) W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature* **373**:347-349 https://doi.org/10.1038/373347a0 | PubMed

19. **Starr ME,** Steele AM, Saito M, Hacker BJ, Evers BM, Saito H (2014) A New Cecal Slurry Preparation Protocol with Improved Long-Term Reproducibility for Animal Models of Sepsis. *Plos One* **9**:e115705 https://doi.org/10.1371/journal.pone.0115705 | PubMed

20. **Zhou JS,** Xing W, Friend DS, Austen KF, Katz HR (2007) Mast cell deficiency in KitW-sh mice does not impair antibody-mediated arthritis. *J Exp Medicine* **204**:2797-2802 https://doi.org/10.1084/jem.20071391 | PubMed

21. **Nigrovic PA,** Gray DHD, Jones T, Hallgren J, Kuo FC, Chaletzky B, Gurish M, Mathis D, Benoist C, Lee DM (2008) Genetic Inversion in Mast Cell-Deficient Wsh Mice Interrupts Corin and Manifests as Hematopoietic and Cardiac Aberrancy. *Am J Pathology* **173**:1693-1701 https://doi.org/10.2353/ajpath.2008.080407 | PubMed

22. **Chen L,** Zhao Y, Lai D, Zhang P, Yang Y, Li Y, Fei K, Jiang G, Fan J (2018) Neutrophil extracellular traps promote macrophage pyroptosis in sepsis. *Cell Death Dis* **9**:597 https://doi.org/10.1038/s41419-018-0538-5 | PubMed

23. **Gomez-Pinilla PJ,** Farro G, Giovangiulio MD, Stakenborg N, Némethova A, Vries A. de, Liston A, Feyerabend TB, Rodewald H.-R, Rodewald H.-R, *et al.* (2014) Mast Cells Play No Role in the Pathogenesis of Postoperative Ileus Induced by Intestinal Manipulation. *Plos One* **9**:e85304 https://doi.org/10.1371/journal.pone.0085304 | PubMed

24. **Dahdah A,** Gautier G, Attout T, Fiore F, Lebourdais E, Msallam R, Daëron M, Monteiro RC, Benhamou M, Charles N, *et al.* (2014) Mast cells aggravate sepsis by inhibiting peritoneal macrophage phagocytosis. *J. Clin. Investig* **124**:4577-4589 https://doi.org/10.1172/jci75212 | PubMed

25. **Thakurdas SM,** Melicoff E, Sansores-Garcia L, Moreira DC, Petrova Y, Stevens RL, Adachi R (2007) The Mast Cell-restricted Tryptase mMCP-6 Has a Critical Immunoprotective Role in Bacterial Infections*. *J Biol Chem* **282**:20809-20815 https://doi.org/10.1074/jbc.m611842200 | PubMed

26. **Young TM,** Bray AS, Nagpal RK, Caudell DL, Yadav H, Zafar MA (2020) Animal Model To Study Klebsiella pneumoniae Gastrointestinal Colonization and Host-to-Host Transmission. *Infect. Immun* **88**:e00071-20 https://doi.org/10.1128/iai.00071-20 | PubMed

27. **Plum T,** Wang X, Rettel M, Krijgsveld J, Feyerabend TB, Rodewald H.-R (2020) Human Mast Cell Proteome Reveals Unique Lineage, Putative Functions, and Structural Basis for Cell Ablation. *Immunity* **52**:404-416.e5. https://doi.org/10.1016/j.immuni.2020.01.012 | PubMed

28. **Gordon JR,** Galli SJ (1991) Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. *J. Exp. Med* **174**:103-107 https://doi.org/10.1084/jem.174.1.103 | PubMed

29. **Adelman MW,** Woodworth MH, Langelier C, Busch LM, Kempker JA, Kraft CS, Martin GS (2020) The gut microbiome's role in the development, maintenance, and outcomes of sepsis. *Crit. Care* **24**:278 https://doi.org/10.1186/s13054-020-02989-1 | PubMed

30. **Fay KT,** Ford ML, Coopersmith CM (2017) The intestinal microenvironment in sepsis. *Biochim. Biophys. Acta (BBA) - Mol. Basis Dis* **1863**:2574-2583 https://doi.org/10.1016/j.bbadis.2017.03.005 | PubMed

31. **Schaubeck M,** Clavel T, Calasan J, Lagkouvardos I, Haange SB, Jehmlich N, Basic M, Dupont A, Hornef M, Bergen M. von, *et al.* (2016) Dysbiotic gut microbiota causes transmissible Crohn's disease-like ileitis independent of failure in antimicrobial defence. *Gut* **65**:225 https://doi.org/10.1136/gutjnl-2015-309333 | PubMed

32. **Muijlwijk GH. van,** Rice TA, Flavell RA, Palm NW, Zoete MR. de (2023) Allobaculum mucilyticum sp. nov. and Allobaculum fili sp. nov., isolated from the human intestinal tract. *Int. J. Syst. Evol. Microbiol* **73** https://doi.org/10.1099/ijsem.0.005635 | PubMed

33. **Jalanka J,** Cheng J, Hiippala K, Ritari J, Salojärvi J, Ruuska T, Kalliomäki M, Satokari R (2020) Colonic Mucosal Microbiota and Association of Bacterial Taxa with the Expression of Host Antimicrobial Peptides in Pediatric Ulcerative Colitis. *Int. J. Mol. Sci* **21**:6044 https://doi.org/10.3390/ijms21176044 | PubMed

34. **Pisani A,** Rausch P, Bang C, Ellul S, Tabone T, Cordina CM, Zahra G, Franke A, Ellul P (2022) Dysbiosis in the Gut Microbiota in Patients with Inflammatory Bowel Disease during Remission. *Microbiol. Spectr* **10**:e00616-22 https://doi.org/10.1128/spectrum.00616-22 | PubMed

35. **Rittirsch D,** Huber-Lang MS, Flierl MA, Ward PA (2009) Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc* **4**:31-36 https://doi.org/10.1038/nprot.2008.214 | PubMed

36. **Cuenca AG,** Delano MJ, Kelly Scumpia KM, Moldawer LL, Efron PA (2010) Cecal Ligation and Puncture. *Curr Protoc Immunol* **91**:19.13.1-19.13.11 https://doi.org/10.1002/0471142735.im1913s91 | PubMed

Peer reviews

Reviewer #1 (Public review):

Summary:

Mast cells have previously been reported to play an important role in bacterial immune defence and act protectively in sepsis. However, many of these findings were based on studies using Kit mutant mice. In this study, the authors conducted a detailed investigation using mast cell-deficient Cpa3 Cre-Master mice. As a result, the authors found that the Cpa3 Cre-Master mice exhibited responses similar to wild-type mice in terms of bacterial immune defense. This suggests that the observed phenotype is not due to mast cell-dependent bacterial immune defense, but rather is associated with dysbiosis of the gut microbiota.

Strengths:

Mast cells have long been reported to play an important role in the protective response against sepsis, and their function in infection defense has been demonstrated. However, Kit mutant mice have been reported to exhibit impaired peristalsis, and several mast cell-specific genetically modified mouse lines have since been developed and examined in detail. This study presents an important finding by logically demonstrating that the exacerbation of sepsis in Kit mice is due to alterations in the gut microbiota, and that the phenotype previously thought to be mast cell-dependent was, in fact, not.

In addition, the experiments were carefully designed using mice with matched genetic backgrounds. These findings underscore the importance of microbiota composition in interpreting immune phenotypes and highlight the need for co-housing controls in mutant mouse studies.

A major strength of this work is the robustness of the CLP data, generated over eight years by three independent researchers across two institutions with large sample sizes, lending strong support to the conclusions.

Weaknesses:

The study assesses only a limited subset of gut bacterial species, leaving the extent to which E. coli expansion contributes to the observed phenotype unclear. Moreover, in the cohousing experiments, there is no evidence provided to confirm successful microbiota normalization between groups. A more detailed analysis of the microbial composition would be necessary to strengthen the reliability of the findings.

It is also important to note that Cpa3-deficient mice exhibit not only mast cell depletion but also defects in basophils and T cells. These additional immunological alterations may counterbalance one another, potentially masking phenotypic changes and complicating interpretation.

Furthermore, it remains to be determined whether the altered gut microbiota observed in KitW/Wv mice is a consequence of impaired intestinal motility, whether a similar phenotype is observed in KitW-sh/W-sh mice, and whether comparable results occur in SCF-deficient models. Addressing these questions would provide greater clarity on the contribution of mast cells versus secondary factors in the observed phenotypes.

Given that KitW/Wv mice exhibit impaired peristalsis, is the observed increase in E. coli a consequence of this dysfunction?

Previous studies with BMMC reconstitution experiments have indicated that mast cells are a source of TNF-how does this align with the current findings?

Comments on revised version.

The authors have made substantial efforts to address the concerns raised in the previous review, and the revised manuscript has been substantially strengthened by the additional microbiome analyses. In particular, the new data provide a more comprehensive characterization of the intestinal microbiota in both Kit mutant and mast cell-deficient mice and strengthen the interpretation that the microbiota alterations observed in Kit mutant mice are associated with Kit deficiency rather than mast cell deficiency per se. Although Enterobacteriaceae were significantly increased based on the unadjusted Welch's t-test, this difference did not remain statistically significant after correction for multiple comparisons. The authors appropriately acknowledge this limitation in the revised manuscript. Overall, I consider the major concerns regarding the microbiome analysis to have been adequately addressed.

https://doi.org/10.7554/eLife.106789.2.sa2

Reviewer #2 (Public review):

Summary:

The authors showed that the high susceptibility to CLP sepsis of Kit-mutant mice is not due to mast cell deficiency, but to dysbiosis.

Recommendations:

(1) The authors showed that E. coli increases in the cecum of Kit-mutant mice, which causes high CLP susceptibility. However, they did not provide any evidence E. coli is responsible for the high susceptibility. In the Figure 3 experiments, the authors administered the same number of cecal bacteria and did not show the number of E. coli after the administration. The authors should provide evidence showing that depletion of E. coli decreases susceptibility.

(2) The author should provide direct evidence of dysbiosis by, for example, shotgun sequencing of cecal and fecal contents.

(3) In case the authors find dysbiosis, they should analyze the mechanisms by which Kit mutation causes dysbiosis.

Comments on revised version.

The revised manuscript focuses on refuting the notion that mast cells play important roles in sepsis. The reviewer agrees with this claim.

https://doi.org/10.7554/eLife.106789.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *Mast cells have previously been reported to play an important role in bacterial immune defense and act protectively in sepsis. However, many of these findings were based on studies using Kit mutant mice. In this study, the authors conducted a detailed investigation using mast cell-deficient Cpa3 Cre-Master mice. As a result, the authors found that the Cpa3 Cre-Master mice exhibited responses similar to wildtype mice in terms of bacterial immune defense. This suggests that the observed phenotype is not due to mast cell-dependent bacterial immune defense, but rather is associated with dysbiosis of the gut microbiota.*
>
> *Strengths:*
>
> *Mast cells have long been reported to play an important role in the protective response against sepsis, and their function in infection defense has been demonstrated. However, Kit mutant mice have been reported to exhibit impaired peristalsis, and several mast cell-specific genetically modified mouse lines have since been developed and examined in detail. This study presents an important finding by logically demonstrating that the exacerbation of sepsis in Kit mice is due to alterations in the gut microbiota, and that the phenotype previously thought to be mast cell-dependent was, in fact, not.*
>
> *In addition, the experiments were carefully designed using mice with matched genetic backgrounds. These findings underscore the importance of microbiota composition in interpreting immune phenotypes and highlight the need for cohousing controls in mutant mouse studies.*
>
> *A major strength of this work is the robustness of the CLP data, generated over eight years by three independent researchers across two institutions with large sample sizes, lending strong support to the conclusions.*
>
> *Weaknesses:*
>
> *The study assesses only a limited subset of gut bacterial species, leaving the extent to which E. coli expansion contributes to the observed phenotype unclear.*

We now performed 16S rRNA sequencing of cecal samples isolated from $Kit^{W/Wv}$ and $Cpa3^{Cre/+}$ mice and their respective littermates. Results are display in a new Figure 4. Our comparative analysis of the cecal microbial communities in $Kit^{W/Wv}$ and $Kit^{+/+}$ mice (Figure 4A+B) confirmed the expansion of *E. coli* (*Enterobacteriaceae*) that we had observed by CFU counts (Figure 3D). Furthermore, it revealed a dysbiotic shift marked by increased abundance

of *Peptostreptococcaceae*, *Verrucomicrobiaceae*, *Coriobacteriaceae*, and *Erysipelotrichaceae* in Kit*W/Wv* mice.

None of these changes was observed when comparing the cecal microbiomes of Cpa3*Cre/+* and Cpa3+/+ mice (Figure 4C+D), indicating that the compositional shift in $Kit^{W/Wv}$ mice is due to the deficiency in Kit but not mast cells. Of note, as stated on page 14, the microbial changes that we observed in $Kit^{W/Wv}$ mice resemble dysbiotic patterns reported in chronic intestinal inflammation, experimental colitis, and impaired barrier function. These new findings fully align with and further support our earlier conclusion that $Kit^{W/Wv}$ mice harbour pro-pathogenic microbiota.

The new results are display in a new Figure 4, and described on pages 9-10 and discussed on pages 13-14.

> *Moreover, in the cohousing experiments, there is no evidence provided to confirm successful microbiota normalization between groups.*

It is correct that we have no direct data to confirm microbiota normalization between groups after co-housing. We note, however, that co-housing is a generally accepted method for microbiota equalization or conversion (Caruso et al., Cell Rep. 2019, Ridaura et al., Science 2013, and reviewed in Moore et al., Clin. Transl. Immunol. 2016). In any case, $Kit^{W/Wv}$ mutants were made resistant to CLP by co-housing. Similar microbiota sequencing results between groups, while useful, would again only be correlative.

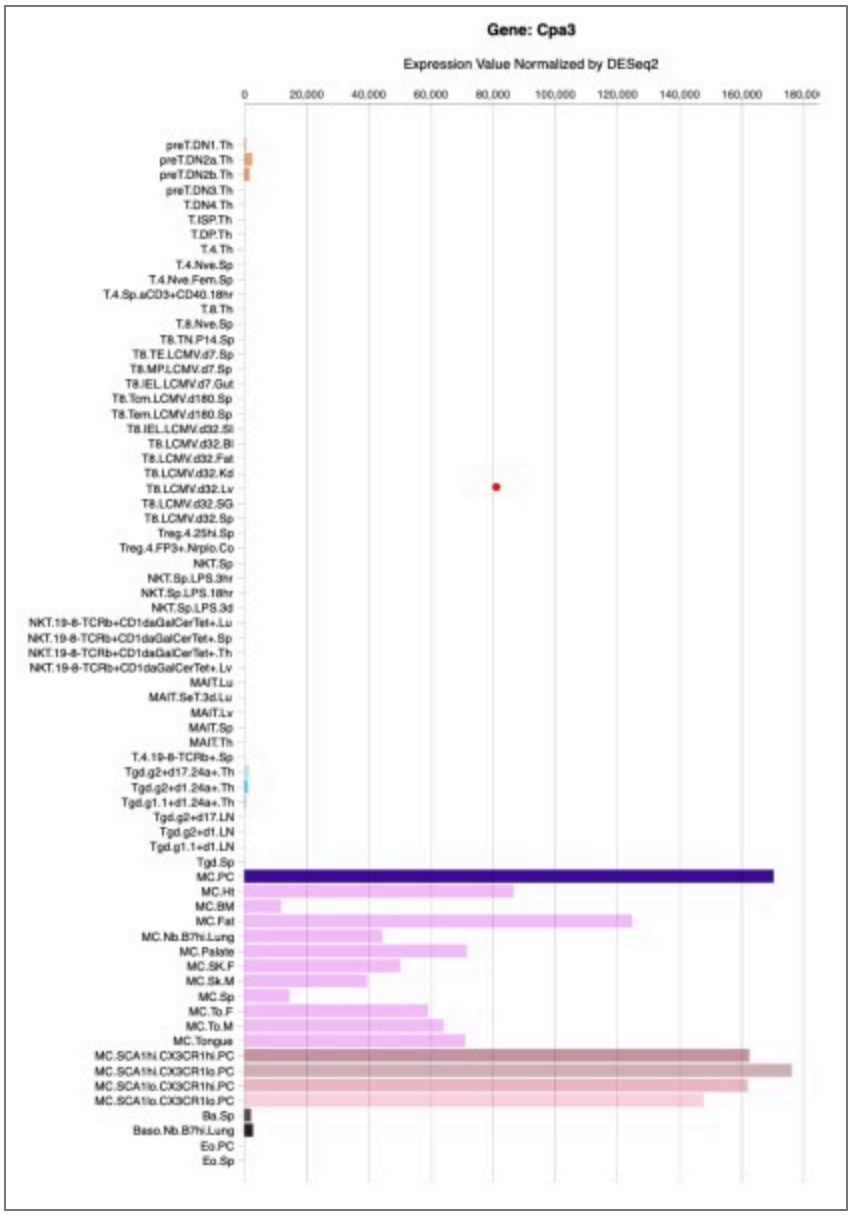
> *A more detailed analysis of the microbial composition would be necessary to strengthen the reliability of the findings.*

See above the new data from 16S rRNA sequencing.

> *It is also important to note that Cpa3-deficient mice exhibit not only mast cell depletion but also defects in basophils and T cells. These additional immunological alterations may counterbalance one another, potentially masking phenotypic changes and complicating interpretation.*

Regarding basophils in $Cpa3^{Cre/+}$ mice, compared to wild-type mice, basophils are reduced to about 40% of normal (Feyerabend et al., Immunity 2011). In $Kit^{W/Wv}$ mice, compared to wild-type mice, basophils are reduced to about 10% of normal. To our knowledge, there has been no phenotype reported in which a reduction in basophils compensates for the loss for mast cells. Given that $Kit^{W/Wv}$ mice have about threefold lower numbers of basophils and are highly susceptible to sepsis, there is no evidence that a reduction in basophils is protective in mast cell-deficient mice. On the contrary, mice that were normal for mast cells but had their basophils depleted were more susceptible to sepsis (Piliponsky et al., Nat. Immunol. 2019). Hence, basophils appear to be protective, and their reduction increases susceptibility. In light of these data and considerations, there is no evidence for a reduction in basophils to counterbalance the loss of mast cells in $Cpa3^{Cre/+}$ mice.

Regarding T cells, there is no evidence, and there are no reports, that $Cpa3^{Cre/+}$ mice have defects in T cells (Feyerabend et al., Immunity 2011, Feyerabend et al., Cell Metabolism 2016). Cpa3 is weakly and transiently expressed early in the T cell lineage (Feyerabend et al., Immunity 2009; for expression levels in T cells versus mast cells, see Author response image 1). In summary, in contrast to the reviewer's claim, there are no known defects in T cell development or T cell functions in $Cpa3^{Cre/+}$ mice. We think the reviewer needs to provide published evidence for his/her claim that Cpa3-deficient mice exhibit defects in T cells. We as authors are also obliged to support our claims scientifically, and rightfully so.



Author response image 1. Generated from the Immgen database. Shown are RNAseq gene expression levels of diverse T-cell and mast cell populations.

> *Furthermore, it remains to be determined whether the altered gut microbiota observed in KitW/Wv mice is a consequence of impaired intestinal motility, whether a similar phenotype is observed in KitW-sh/W-sh mice, and whether comparable results occur in SCF-deficient models. Addressing these questions would provide greater clarity on the contribution of mast cells versus secondary factors in the observed phenotypes.*

The purpose of our study was to verify or refute the key claim dating back to two 1996 Nature papers that mast cells play important roles against sepsis. We demonstrate here that this is not the case because mice without mast cells ($Cpa3^{Cre/+}$ mice) were as resistant to sepsis as wild-type mice. Hence, mast cells are not involved in the immunity against sepsis, and 'secondary factors' are not involved in this simple experiment (both groups of mice, wild-type and $Cpa3^{Cre/+}$ mice, were on the identical genetic background). Second, $Kit^{W/Wv}$ mice are also as resistant to sepsis as wild-type mice when confronted with the identical intestinal slurry. Therefore, $Kit^{W/Wv}$ mice have no immune deficit in response to sepsis. Hence, in our view, the underlying immunological question regarding the role of mast cells in sepsis has been

conclusively addressed and answered by our data. We have changed the title to emphasize this central question.

The reviewer now asks us to delve even deeper into Kit biology and in particular intestinal pathophysiology in this and other Kit or steel mutants. While we share his/her interest in such questions, we fully disagree with the statement that 'addressing these questions would provide greater clarity on the contribution of mast cells versus secondary factors in the observed phenotypes.' We do not intend to enter the field of gut physiology or its link to microbiota, all the more because any results would not affect the central conclusion of our manuscript.

> *Given that KitW/Wv mice exhibit impaired peristalsis, is the observed increase in E. coli a consequence of this dysfunction?*

See above

> *Previous studies with BMMC reconstitution experiments have indicated that mast cells are a source of TNF - how does this align with the current findings?*

It does not align well. It is possible that cultured and transplanted mast cells (BMMC) produce TNF. Given that we did not find a reduction in TNF levels in the peritoneal lavage or serum in mice without mast cells undergoing sepsis, under physiological conditions mast cell-derived TNF does not seem to have a measurable impact on total TNF levels.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *This study presents a useful finding that the high susceptibility to CLP sepsis of Kitmutant mice is not due to mast cell deficiency, but to dysbiosis.*
>
> *However, the present data are insufficient and incomplete to support the conclusion, and would benefit from more rigorous approaches. With the mechanism part strengthened, this paper would be of interest to researchers on mast cell biology and mucosal immunology.*

We disagree with the view that our data are insufficient and incomplete. Our results demonstrate that mice lacking mast cells ($Cpa3^{Cre/+}$ mice) are as resistant to sepsis as wild-type mice, demonstrating that mast cells do not play a detectable role in immunity against sepsis. Additionally, we show that $Kit^{W/Wv}$ mice exhibit the same resistance to sepsis as wild-type mice when confronted with the identical intestinal slurry. This finding demonstrates that $Kit^{W/Wv}$ mice have no immune deficit in response to sepsis. These central data are both sufficient and complete, given that our data fully address the potential role of mast cells in sepsis. Our study aimed to investigate the role of mast cells in sepsis, not to examine the mechanisms of dysbiosis or associated pathological phenotypes in Kit-mutant controls. We have changed the title to make this point.

> *Recommendations:*
>
> *(1) The authors showed that E. coli increases in the cecum of Kit-mutant mice, which causes high CLP susceptibility. However, they did not provide any evidence E. coli is responsible for the high susceptibility.*

We showed that *E. coli* CFUs were increased in the cecum of Kit-mutant mice, but we did not state that this causes CLP susceptibility. We wrote: 'Hence, $Kit^{W/Wv}$ microbiota contains high levels of *E. coli*, which may underlie the observed pathogenicity'. We demonstrated that intestinal slurry from $Kit^{W/Wv}$ mice is more pathogenic compared to intestinal slurry from wild-type mice. However, we did not search for or identify the bacterial species that causes

this increased pathogenicity because we were addressing the role of mast cells in sepsis. We demonstrate an association of pathogenicity in sepsis experiments with cecal content of pathogenic bacteria (see also the new data on 16S rRNA sequencing). The same argument could be made for each bacterial species identified but this would be very complex experiments (both microbiologically and immunologically) given requirements for bacterial isolation, titration, and considerations of synergism. We therefore refrain from this undertaking.

> *In the Figure 3 experiments, the authors administered the same number of cecal bacteria and did not show the number of E. coli after the administration.*

The samples were split and one aliquot was analysed by microbiology and the other aliquot was injected intraperitoneally. Fig. 3d shows the colony-forming units (for *Lactobacilli* and *E coli*) from aliquots of cecal slurry used in the intraperitoneal injection experiments shown in Fig. 3a-c. Hence, our data show the colony-forming units that were injected into the mice. It is unclear to us why this is not the key information rather than 'the number of *E. coli* after the administration'.

> *The authors should provide evidence showing that depletion of E. coli decreases susceptibility.*

See response to point 1 above.

> *(2) The author should provide direct evidence of dysbiosis by, for example, shotgun sequencing of cecal and fecal contents.*

We performed 16S rRNA sequencing of cecal contents and observed a dysbiotic shift towards an increase of *Peptostreptococcaceae*, *Verrucomicrobiaceae*, *Coriobacteriaceae*, *Enterobacteriaceae*, and *Erysipelotrichaceae* in $Kit^{W/Wv}$ mice compared to $Kit^{+/+}$ controls. None of these changes was observed when comparing the cecal microbiomes of $Cpa3^{Cre/+}$ and $Cpa3^{+/+}$ mice, indicating that the compositional shift in $Kit^{W/Wv}$ mice is due to deficiency in Kit but not mast cells. Of note, as stated on page 14, the microbial changes we observed in $Kit^{W/Wv}$ mice resemble dysbiotic patterns reported in chronic intestinal inflammation, experimental colitis, and impaired barrier function.

These new findings fully align and further support with our earlier conclusion that $Kit^{W/Wv}$ mice harbour pro-pathogenic microbiota.

The new results are display in a new Figure 4, and described on pages 10-11 and discussed on pages 13-14.

> *(3) In case the authors find dysbiosis, they should analyze the mechanisms by which Kit mutation causes dysbiosis.*

We have no intention to further explore Kit biology and in particular the intestinal pathophysiology caused by the Kit mutation because any results would not affect the central conclusion of our manuscript (see title). The review process and the revision shall center on making the core of a paper as conclusive as possible, and not widen a paper by requests 'tangential to the main conclusion' (Kaelin Jr. Nature 2017).

References:

Caruso, R., Ono, M., Bunker, M. E., Núñez, G. & Inohara, N. Dynamic and Asymmetric Changes of the Microbial Communities after Cohousing in Laboratory Mice. Cell Rep. 27, 3401-3412.e3 (2019).

Feyerabend, T. B. et al. Deletion of Notch1 Converts Pro-T Cells to Dendritic Cells and Promotes Thymic B Cells by Cell-Extrinsic and Cell-Intrinsic Mechanisms. Immunity 30, 67–79

(2009).

Feyerabend, T. B. et al. Cre-Mediated Cell Ablation Contests Mast Cell Contribution in Models of Antibody- and T Cell-Mediated Autoimmunity. Immunity 35, 832–844 (2011).

Feyerabend, T. B., Gutierrez, D. A. & Rodewald, H.-R. Of Mouse Models of Mast Cell Deficiency and Metabolic Syndrome. Cell Metab 24, 1–2 (2016).

Kaelin Jr, W. G. Publish houses of brick, not mansions of straw. Nature 545, 387– 387 (2017).

Moore, R. J. & Stanley, D. Experimental design considerations in microbiota/inflammation studies. Clin. Transl. Immunol. 5, e92 (2016).

Piliponsky, A. M. et al. Basophil-derived tumor necrosis factor can enhance survival in a sepsis model in mice. Nat. Immunol. 20, 129–140 (2019).

Ridaura, V. K. et al. Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice. Science 341, 1241214 (2013).

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *Suggestions for improved or additional experiments, data, or analyses:*
>
> *(1) The study examines only a limited range of gut bacterial species, making it difficult to determine the specific contribution of E. coli expansion to the observed phenotype. A more comprehensive microbial profiling (e.g., 16S rRNA sequencing or metagenomics) would significantly strengthen the conclusions (e.g., Cpa3-mast cell deficient mice, Kit WWv, Kit W-sh/W-sh mice).*

As mentioned above, we now performed 16S rRNA sequencing of cecal contents from $Kit^{W/Wv}$ and $Cpa3^{Cre/+}$ mice and their respective littermates. Addressing microbiota in $Kit^{W\text{-}sh/W\text{-}sh}$ mice would not add information relevant for our paper.

> *(2) The role of impaired peristalsis in KitW/Wv mice as a contributor to microbial dysbiosis and increased E. coli burden should be further explored. Complementary studies using KitW-sh/W-sh or SCF-deficient mice could clarify whether the observed microbiota changes are unique to the W/Wv model.*

We changed the title of the manuscript to emphasize our (unchanged) focus on the immunological role of mast cells in protecting against bacterial sepsis. The responses of $Cpa3^{Cre}$ mice clearly ruled out a role of mast cells to these infectious conditions. Our observation of altered microbiota in $Kit^{W/Wv}$ mice is consistent with their increased CLP susceptibility. We cite the known peristalsis deficit of $Kit^{W/Wv}$ mice as a possible explanation for the microbiota alterations. In the future, other investigators may find it interesting to elucidate the link between Kit mutations and dysbiosis. As stated further above, in our view, these additional questions and potential data have no bearings on the conclusions of our paper.

> *(3) In the cohousing experiments, no data are provided to confirm whether microbiota normalization was achieved between groups. Including microbial composition data pre- and post-cohousing would improve the reliability of the interpretation.*

Cohousing made the susceptibility of $Kit^{W/Wv}$ and $Kit^{+/+}$ mice comparable. Detailed analysis of the extent of microbiota normalization would only make sense to ultimately determine specific taxa or combinations thereof that are responsible for the increased susceptibility of $Kit^{W/Wv}$ mice, a question that was never the goal of this study.

> *(4) The use of Cpa3 Cre/+ mice introduces potential confounders, as these mice also have defects in basophils and T cells. Functional validation or additional models (e.g., Mas-TRECK or Mcpt5-Cre mice) could help isolate the mast cell-specific effects.*

See our detailed explanation above (Reviewer #1 Public review). It is incorrect to claim that $Cpa3^{Cre/+}$ mice have defects in T cells. The reviewer needs to provide published evidence for his/her claim that Cpa3-deficient mice exhibit defects in T cells. We as authors are also obliged to support our claims scientifically, and rightfully so.

> *(5) Clarification is needed regarding the role of mast cell-derived TNF. Given previous reports using BMMC reconstitution that implicate mast cells as a source of TNF, reconciling these findings with the current study's results would strengthen interpretation.*

We also disagree here. Experiments in normal unmanipulated mice are inevitably superior to Kit mutants after BMMC reconstitution which is an artificial system. The transplanted cells do not mature normally and don't settle in their natural niches. We mentioned in the discussion that there is conflicting literature derived from different models and mice.

But we do not share the expectation that results obtained with Kit-independent models, that differ from previous studies using Kit mutants, necessarily require reconciliation. The experiments are simply incomparable and the most physiologically relevant experiment will pave the way. Research on mast cell functions based on BMMC-reconstituted Kit mutants has meanwhile proven to be unreliable.

> *(6) A clearer delineation between mast cell-dependent and microbiota-mediated mechanisms in the discussion sections would enhance readability and impact.*

We restructured the discussion and distinguished between Kit-dependent and mast cell-dependent phenotypes. We also discussed in detail the observed microbiota differences and their influences for the outcomes in the different sepsis experiments.

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