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Histamine $H_2$ Receptor Signaling in the Pathogenesis of Sepsis: Studies in a Murine Diabetes Model

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Type 1 diabetes enhances susceptibility to infection and favors the sepsis development. In addition, diabetic mice produced higher levels of histamine in several tissues and in the blood after LPS stimulation than nondiabetic mice. In this study, we aimed to explore the role of mast cells (MCs) and histamine in neutrophil migration and, consequently, infection control in diabetic mice with mild sepsis (MS) induced by cecum ligation and puncture. We used female BALB/c, MC-sufficient (WB/B6), MC-deficient (W/W$^v$), and NOD mice. Diabetic mice given MS displayed 100% mortality within 24 h, whereas all nondiabetic mice survived for at least 5 d. The mortality rate of diabetic mice was reduced to 57% after the depletion of MC granules with compound 48/80. Moreover, this pretreatment increased neutrophil migration to the focus of infection, which reduced systemic inflammatory response and bacteremia. The downregulation of CXCR2 and upregulation of G protein–coupled receptor kinase 2 in neutrophils was prevented by pretreatment of diabetic mice given MS with compound 48/80. In addition, blocking the histamine $H_2$ receptor restored neutrophil migration, enhanced CXCR2 expression, decreased bacteremia, and improved sepsis survival in alloxan-induced diabetic and spontaneous NOD mice. Finally, diabetic W/W$^v$ mice had neutrophil migration to the peritoneal cavity, increased CXCR2 expression, and reduced bacteremia compared with diabetic WB/B6 mice. These results demonstrate that histamine released by MCs reduces diabetic host resistance to septic peritonitis in mice. The Journal of Immunology, 2013, 191: 000–000.

Sepsis is a common and serious complication in diabetic patients, and is a well-recognized cause of mortality (1, 2). Mice given experimental diabetes are more susceptible to a variety of bacterial infections, including *Klebsiella pneumoniae* (3), *Listeria monocytogenes* (4), *Streptococcus* sp. (5), and *Mycobacterium tuberculosis* (6). Despite these considerations, little is known about the immunological mechanisms that confer the increased susceptibility to infection observed during diabetes (7, 8). Deficiencies in leukocyte functions have been described in experimental and clinical diabetes, including reduced migration (9), chemotaxis (10), phagocytosis (11), and microbicidal activity (12). We and others have shown that the severity of sepsis in nondiabetic mice and patients is associated with reduced neutrophil migration to the focus of infection. This reduction in neutrophil migration is due to the internalization of the chemokine receptor CXCR2 and modulation of adhesion molecules expression on endothelium (13–15). CXCR2 internalization is associated with upregulation of G protein–coupled receptor kinase 2 (GRK2) expression in circulating neutrophils, which is, at least partially, mediated by systemic TLR2 and TLR4 activation (16–18).

Invading microorganisms are recognized via pattern recognition molecules such as TLRs by resident cells, including mast cells (MCs) and macrophages (19). TLR2 and TLR4 signaling triggers a local inflammatory response through cytokine production and MC degranulation (20). MC-deficient mice (W/W$^v$) are more susceptible to peritonitis induced by either cecal ligation and puncture (CLP) or *Klebsiella pneumonia* infection, and this susceptibility can be suppressed by improving the local inflammatory response by transferring MCs into the peritoneal cavity (21, 22). On the other hand, systemic release of cytokines by MCs impairs neutrophil migration to the focus of infection and can lead to death during sepsis (23, 24).

Histamine, a major component of MC granules, binds to four receptor subtypes, histamine $H_1$ receptor ($H_1$) to $H_4$. Neutrophils have been shown to express both $H_1$ and $H_2$ (25, 26), and $H_2$ signaling mediates the inhibition of neutrophil chemotaxis (27). In addition, histamine signaling via $H_2$ negatively regulates human neutrophil activation, superoxide production, degranulation, and 5-lipoxygenase–derived leukotriene B4 (LTB4) synthesis (28–30). In the CLP model, 5-lipoxygenase–null mice with severe sepsis (SS) presented a reduction in neutrophil migration and increase of bacterial load in the focus of infection (31). Collectively, these findings imply that histamine could inhibit the LTB4 production and dampen the neutrophil migration.
It was demonstrated that histamine levels are increased in plasma and tissues in diabetic rats, as well as in patients with diabetes mellitus (32, 33). Moreover, levels of histamine are higher in diabetic rats than in nondiabetic animals after LPS administration (34). In agreement, in this study, we found that histamine is increased in diabetic mice undergoing septic peritonitis. Moreover, we observed that histamine released by MCs decreases neutrophil migration to the infection site through interaction with H2, resulting in impaired host defense and a poor sepsis outcome in diabetic mice.

Materials and Methods

Mice

Female BALB/c, MC-sufficient (WB/B6), W/Wv, and NOD mice were housed in facilities of Department of Pharmacology, School of Medicine of Ribeirão Preto at 23–25°C with free access to water and food. Animal care and treatment was based on the Guide for the Care and Use of Laboratory Animals (35), and all experiments were approved by the Animal Research Ethics Committee of the School of Medicine of Ribeirão Preto (no. 001/2008).

Diabetes induction

Diabetes was induced with a single i.v. injection of alloxan (Sigma Chemical Company, St. Louis, MO) at a dosage of 50 mg/kg in saline (100 μL). The nondiabetic control group received an i.v. injection of saline alone. Blood samples were collected from the tail vein to measure blood glucose levels using a glucometer (Precision Xtra Abbott, Alameda, CA).

Sepsis model

Sepsis was induced by CLP as previously described (36). In brief, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) by i.p. injection. A 1-cm midline incision was made on the anterior abdomen, and the cecum was exposed and ligated below the ileocecal junction without causing bowel obstruction. A single puncture was made in the cecum using an 18-gauge needle to induce SS, or three punctures were made using a 30-gauge needle to induce mild sepsis (MS). The cecum was squeezed to express its contents through the puncture holes and then placed back into the abdominal cavity. The peritoneal wall and skin incisions were closed. Sham-operated (Sham) animals were submitted to laparotomy but without punctures in cecum.

Pharmacological treatment

Nondiabetic mice were treated s.c. with histamine (3 μg or 10 μg in 100 μl saline) (Sigma Chemical Company, St. Louis, MO), 1 h before CLP and every 24 h afterward for a total of 5 d for survival rate analysis, or treated 1 h before CLP with 10 μg histamine per mouse for neutrophil migration, bacterial load, and CXCR2 expression evaluation. Ten days after alloxan treatment, diabetic mice were injected i.p. with pyrillamine (20 mg/kg, a selective H1 antagonist), cimetidine (10 mg/kg, a selective H2 antagonist), imidazole (5 mg/kg, an H2/4 antagonist), or saline 1 h before CLP and every 2 h afterward for a total of 5 d for the survival rate analysis. Compound 48/80 (C48/80; Sigma Chemical Company) was administered two times per day for 4 d i.p. at dosages of 0.6, 1.0, 1.2, or 2.4 mg/kg before CLP. Ten days after saline or alloxan administration, diabetic and nondiabetic mice underwent MS induction, SS induction, or a sham operation as the control according to the CLP model (Supplemental Fig. 1A).

Neutrophil migration to the peritoneal cavity

Peritoneal cavity lavage (PCL) was performed after 4 or 6 h of sepsis induction using 3 ml PBS/EDTA 1 mM. Total cell counts were obtained using a cell counter (Coulter Analyzer; Coulter), and differential cell counting was conducted using cytocentrifuge slides (Shandon Southern Products) stained with May-Grünwald-Giemsa.

Bacterial count

Four or 6 h after CLP, the blood was collected and the peritoneal cavity was washed with 1.5 ml sterile PBS/EDTA. Aliquots of serial dilutions of these samples were plated on Muller-Hinton agar dishes (Difco Laboratories) and incubated at 37°C. CFUs were analyzed after 18 h, and the results were expressed as log of CFU/10 μl.

GRK2 immunofluorescence

Immunofluorescence was used to analyze GRK2 expression on blood neutrophils at 6 h postsurgery using anti-mouse GRK2 (Y137; Abcam) as the primary Ab, as previously described (14). Cell nuclei were stained with DAPI. Fluorescent images were analyzed using an Olympus BX-50 epifluorescence microscope.

Flow cytometry

One hundred microliters of blood was stained using PerCP-Cy5.5-conjugated anti-mouse myeloid lineage differentiation Ag Gr-1 (GR-1) (BD Bioscience, Franklin Lakes, NJ), PE-conjugated anti-CXCR2 mAb (R&D Systems, Minneapolis, MN), or the appropriate isotype controls. The cells were washed, fixed in 2% formaldehyde, and analyzed by flow cytometry in a FACSort flow cytometer using CellQuest software. Neutrophils were identified by their light scatter properties and high GR-1 expression. MC number was determined using double staining with PE-Cy5-conjugated anti-CD117 and FITC-conjugated anti-FcεRI (eBioscience, San Diego, CA). The GRK2 staining was evaluated by flow cytometry in neutrophils as previously described (37).

Detection of serum TNF-α and CXCL-2 levels

TNF-α and CXCL-2 levels were assessed in serum and PCL 6 h after surgery by ELISA. The results were displayed in picograms per milliliter as the mean ± SEM.

Histamine measurement

Mouse plasma histamine concentration was measured 6 h after surgery using an immunoenzymatic method with a manufactured kit (Cayman, Ann Arbor, MI).

Neutrophil isolation and chemotaxis assay

Bone marrow or blood neutrophils were isolated using a Percoll gradient as described previously (14). Neutrophil suspensions were incubated with RPMI 1640 (Sigma-Aldrich) or histamine at a concentration of 1 or 10 μM for 1 h, and neutrophil chemotaxis was assessed toward CXCL-2 (30 ng/ml; R&D Systems, Minneapolis, MN) in a Boyden chamber (Neuro Probe, Bethesda, MD). In another set of experiments, the chemotactic activity of blood neutrophils obtained from related experimental groups was assessed for CXCL-2 (30 ng/ml), fMLF (10−7 M), or RPMI 1640 (negative control).

Vascular leakage

Evans blue dye (2.5% in 100 μl of PBS) was i.v. administered 30 min before CLP. Two hours after CLP, the mice were killed, and the peritoneal cavity was washed with 1.5 ml PBS/EDTA (1 mM). The cells were spun down, and the OD of the supernatant at 600 nm was measured. The concentration of Evans blue in peritoneal exudates was determined under a standard curve.

Determination of the myeloperoxidase activity

The extent of leukocyte accumulation in the lung was measured by myeloperoxidase assay as previously described (38). The absorbance of the tissue supernatant was compared with a standard curve of mouse peritoneal neutrophils, and the results were reported as the mean ± SEM of the total number of neutrophils per milligram of lung.

Statistical analysis

The log-rank test was used to evaluate the survival rate, and the proportional risk model of Cox was used to identify the differences between groups. The Mann–Whitney U test was used to assess the following variables: blood and PCL bacterial count between vehicle-administered MS group versus histamine-administered MS group and body weight. The one-way ANOVA, followed by Tukey’s multiple-comparison test, was used for other variables. A p value < 0.05 was considered statistically significant.

Results

Diabetic mice showed increased MC numbers after MS

Alloxan-induced diabetes is a common experimental model used to study the pathophysiologic mechanisms involved in type 1 diabetes (39). Blood glucose levels in diabetic mice were increased 10 d after alloxan administration (Supplemental Fig. 1B), and these mice showed reduced body weight compared with nondiabetic mice (Supplemental Fig. 1C). We also evaluated the numbers of MCs (FceRIε/CD117+ cells) in the peritoneal cavity during diabetes by flow cytometry. Interestingly, we observed higher...
numbers of MCs in the peritoneal cavity of sham-operated (sham) diabetic mice compared with sham nondiabetic mice. In addition, diabetic mice had a significant increase in MCs in their peritoneal cavity compared with nondiabetic mice after MS induction. However, the number of MCs in MS diabetic mice did not differ from sham diabetic mice (Fig. 1A). We also observed a significant increase in the MC numbers in the peritoneal cavity of naive diabetic mice that did not undergo sham surgery compared with related nondiabetic mice (Supplemental Fig. 1D).

**MC degranulation contributes to sepsis onset in diabetic mice**

To evaluate the role of MCs in diabetic mice given sepsis, we pretreated diabetic mice with C48/80, an agent that induces MC-granule depletion (40), for 4 d before MS induction. Diabetic mice exhibited 100% mortality rate after MS, whereas all nondiabetic mice survived for 5 d after surgery (Fig. 1B). C48/80 pretreatment of diabetic mice enhanced survival rate to 42% after MS induction. As a control for sepsis induction, we induced SS in nondiabetic mice and observed that they died within 3 d after surgery (Fig. 1B). The nondiabetic mice pretreated with C48/80 had a 33.3% survival rate after SS, whereas all SS mice that were not pretreated with C48/80 died. The survival rates of the pretreated mice and nonpretreated mice after MS were 85.7 and 100%, respectively, demonstrating that the pretreatment with C48/80 did not alter the MS survival rate (Supplemental Fig. 2D). It is important to mention that the pretreatment of diabetic mice with C48/80 did not increase the MS survival rate (Fig. 1C, 1D). MS nondiabetic mice showed a significant increase in neutrophil migration to the peritoneal cavity, which were similar to the numbers observed in SS nondiabetic mice. Notably, pretreatment of diabetic mice with C48/80 significantly prevented the impaired neutrophil migration toward the focus of infection (Fig. 1E). Similarly, the directional migration (chemotaxis) toward CXCL-2 was significantly increased in these pretreated mice compared with MS nontreated diabetic mice (Fig. 1F). We also observed that the pretreatment with C48/80 of diabetic mice restored the random migration (chemokinesis) of blood neutrophils compared with nontreated diabetic mice after MS induction (Fig. 1F).

High systemic levels of proinflammatory cytokines and leukocyte sequestration in the lungs correlate with sepsis severity (41). Accordingly, we found higher levels of leukocytes in the lungs of diabetic MS mice and nondiabetic SS mice than in nondiabetic MS mice. Pretreatment of diabetic mice with C48/80 attenuated this leukocyte infiltration into the lungs after MS induction (Supplemental Fig. 2A). In addition, diabetic MS mice exhibited higher serum concentrations of TNF-α and CXCL-2 than nondiabetic mice.

**FIGURE 1.** MC granule depletion induces septic peritonitis control in diabetic mice. MC numbers were quantified in the peritoneal cavity using specific markers (CD117 and FcεRI) in nondiabetic or diabetic mice 10 d after alloxan administration (A). Nondiabetic mice and diabetic mice were pretreated with saline or C48/80 before induction of MS. SS was carried out in nondiabetic mice. The survival rates were evaluated daily for 5 d after CLP surgery (B). Bacterial burden in the PCL (C), blood (D), and neutrophil numbers (E) into the peritoneal cavity was quantified 6 h after surgery. Blood neutrophils were obtained from sham-operated, nondiabetic (ND), and diabetic mice pretreated with saline or C48/80 after MS surgery. The chemotactic activity of blood neutrophils toward CXCL-2 (30 ng/ml) or RPMI 1640 (negative control) was determined in Boyden chambers (F). The results were expressed as the mean ± SEM (n = 5–7). These are representative graphs of at least two independent experiments. *p ≤ 0.05 compared with the nondiabetic sham-operated group, #p ≤ 0.05 compared with the nondiabetic MS group, &p ≤ 0.05 compared with the diabetic MS group.
abetic MS mice, which were reduced significantly by C48/80 pre-
treatment (Supplemental Fig. 2B, 2C).

**MC degranulation promotes GRK2 upregulation and CXCR2
downregulation in neutrophils**

Reduced neutrophil migration to focus of infection can result from
GRK2-mediated downregulation of CXCR2 in circulating neu-

trophils (16). To address this possibility, we analyzed CXCR2 ex-
pression by flow cytometry and GRK2 by immunofluorescence in

circulating neutrophils after MS in alloxan-induced diabetic and

nondiabetic mice. As shown in Fig. 2A, circulating neutrophils from
MS diabetic mice exhibited a significant reduction in CXCR2 ex-
pression compared with those from both sham (nondiabetic and
diabetic) and MS nondiabetic mice. Pretreatment of diabetic mice
with C48/80 significantly prevented the CXCR2 downregulation in
MS diabetic mice. Moreover, we observed that GRK2 protein ex-
pression was increased in neutrophils from MS diabetic mice and

SS nondiabetic mice compared with the MS nondiabetic or sham-
operated groups. The pretreatment of diabetic mice with C48/80
prevented this increased GRK2 expression (Fig. 2C). Our results
also demonstrated that neutrophils from SS mice presented higher
GRK2 expression compared with sham-operated nondiabetic mice.
In addition, pretreatment with C48/80 prevented GRK2 induction in
the neutrophils from these mice (Supplemental Fig. 2E).

**H2 antagonist treatment alleviates sepsis in diabetic mice**

As shown in Fig. 2B, MS diabetic mice produced higher levels of
histamine compared with both sham (nondiabetic and diabetic)
and MS nondiabetic mice. As previously shown (Fig. 1B), de-

pleting MC granules with C48/80 decreased the severity of sepsis
in diabetic mice after MS induction. This pharmacologic inter-
vention also significantly decreased serum histamine levels in MS
diabetic mice (Fig. 2B). This finding suggested that the high serum
levels of histamine in diabetic mice may contribute to poor sepsis
outcome. To test this hypothesis, we used histamine receptor
antagonists in diabetic mice given MS. Treatment with an H1
antagonist (pyrilamine) delayed the death of the mice, but all mice
still died within 4 d of MS induction (Fig. 3A). In contrast,
treating diabetic mice with an H2 antagonist (cimetidine) in-
creased the survival rate by ~45%. The treatment of MS diabetic
mice with an H3/4 antagonist (thioperamide) did not influence their
survival rate (Fig. 3A). As shown in Fig. 3B, the combination of
H1 and H2 antagonists had no effect on survival rates of diabetic
mice after MS induction.

We next evaluated the effects of the H2 antagonist on neutrophil
recruitment to the focus of infection, blood bacterial load, and
CXCR2 expression in circulating neutrophils in MS diabetic mice.
H2 blockade significantly restored the neutrophil migration to
the peritoneal cavity of MS diabetic mice (Fig. 3C), resulting in
a significantly lower bacterial load in the blood compared with
nontreated MS diabetic mice (Fig. 3D). In addition, treatment with
the H2 antagonist increased CXCR2 expression in the neutrophils
of the MS diabetic mice (Fig. 3E). Blood neutrophils from the MS
diabetic mice have a reduced chemotactic response to CXCL-2,
but not to fMLF, compared with the MS nondiabetic mice, ex-
cluding the possibility of cross desensitization for this receptor. In

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**FIGURE 2.** MC granule depletion prevents CXCR2 internalization and histamine release during polymicrobial peritonitis in diabetic mice. Quantitative
analysis of the mean intensity of fluorescence (MFI) for CXCR2 in blood cells with high expression of GR-1 (neutrophils) was evaluated by flow cytometry
(A). Serum histamine levels were determined 6 h after surgery from several experimental groups as described earlier (B). Blue staining by DAPI represents
neutrophil nuclei; red staining represents GRK2 expression in neutrophil cytoplasm (C) (original magnification ×400). An overlay of nuclei and GRK2
expression shows the qualitative analysis from several experimental groups. Results are expressed as the mean ± SEM (n = 5–7). These are representative
graphs of at least two independent experiments. *p ≤ 0.05 compared with the nondiabetic sham-operated group, **p ≤ 0.05 compared with the nondiabetic
MS group, †p ≤ 0.05 compared with the diabetic sham-operated group, ‡p ≤ 0.05 compared with the diabetic MS group.
addition, H2 pretreatment of the MS diabetic mice significantly restored the chemotactic response to CXCL-2, compared with the chemotactic response of the blood neutrophils of the MS diabetic nontreated mice (Supplemental Fig. 3A).

To determine whether these observations in MS diabetic mice were attributable to differences in vascular permeability between the septic groups, we used Evans blue extravasation in the peritoneal cavity as an index for vascular leakage. The Evans blue content during the first 2 h after MS was prominently increased in both the nondiabetic and diabetic mice compared with nondiabetic sham-operated mice. H2 antagonist treatment did not alter the Evans blue content in the peritoneal cavity of the MS diabetic mice (Fig. 3F).

Histamine is involved in septic peritonitis susceptibility

Our earlier results suggested that histamine negatively regulated neutrophil migration. To test this hypothesis, we first incubated neutrophils with histamine (1 and 10 μM) and observed a reduction in their chemotactic response to CXCL-2 (Fig. 4A). Accordingly, neutrophil incubation with histamine (10 μM) also promoted CXCR2 internalization (Fig. 4B). Pretreatment of neutrophils with an H2 antagonist significantly prevented the histamine-induced CXCR2 downregulation (Fig. 4B). In addition, neutrophils incubated with histamine showed increased intracellular GRK2 expression compared with neutrophils incubated with RPMI (control). However, preincubation of neutrophils with an H2 antagonist significantly diminished the histamine-induced upregulation of GRK2 expression (Supplemental Fig. 3B, 3C).

Enhanced infiltration of neutrophils into the inflammatory site was observed in air-pouch inflammation (42) and bacterial peritonitis (43) in histidine decarboxylase–deficient mice, which cannot produce histamine, indicates that histamine inhibits neutrophil recruitment. Thus, we examined the role of histamine in neutrophil recruitment in the septic peritonitis model. In vivo, administration of 3 or 10 μg histamine per mouse decreased the survival rates by ~30 and 50%, respectively, 5 d after MS surgery (Fig. 4C). Histamine administration also significantly inhibited neutrophil migration to the focus of infection, resulting in impaired infection control as shown by reduced bacterial clearance in the peritoneal cavity and an enhanced bacterial load in the blood (Fig. 4D, 4E). Moreover, systemic histamine administration significantly reduced the CXCR2 expression in the blood neutrophils in MS mice (Fig. 4F). Moreover, the H2 antagonist treatment significantly prevented the downregulation of CXCR2 expression in neutrophils from mice given MS (Fig. 4F).

MC deficiency protects diabetic mice from SS

Next, we evaluated sepsis progression in mice lacking MCs (W/Wv) and their littermate controls (WB/B6). Our results showed that nondiabetic W/Wv mice were more susceptible to MS than control WB/B6 nondiabetic mice (Fig. 5A). We observed a significant decrease in neutrophil numbers in the peritoneal cavity accompanied by increased bacterial load at the focus of infection and in the blood of MS W/Wv nondiabetic mice compared with MS WB/B6 nondiabetic mice (Fig. 5B–D). In contrast, diabetic W/Wv mice with MS showed ~25% improvement in the survival rate compared with WB/B6 diabetic mice with MS (Fig. 5A). In parallel, we observed that diabetic WB/B6 mice given MS had a significant reduction in neutrophil migration and increased peri-
toneal cavity and blood bacterial loads compared with nondiabetic WB/B6 MS mice. In contrast, diabetic W/Wv mice displayed higher neutrophil numbers and reduced bacterial loads in the peritoneal cavity and blood compared with diabetic WB/B6 mice after the MS induction (Fig. 5B–D). Analysis of blood neutrophils by flow cytometry showed that MC deficiency did not affect CXCR2 expression on neutrophils from nondiabetic mice with MS, but prevented CXCR2 downregulation on neutrophils after MS induction in diabetic mice (Fig. 5E).

**H2 antagonist treatment improves infection control in NOD mice**

Finally, we used NOD mice, which spontaneously develop type 1 diabetes, to confirm the role of H2 in sepsis pathogenesis in diabetic mice. We first observed a significant increase in MC numbers (FceRI+/CD117+ cells) in the peritoneal cavity of NOD mice after MS induction (Fig. 5B–D). Analysis of blood neutrophils by flow cytometry showed that MC deficiency did not affect CXCR2 expression on neutrophils from nondiabetic mice with MS, but prevented CXCR2 downregulation on neutrophils after MS induction in diabetic mice (Fig. 5E).

**Discussion**

The factors that determine whether a particular infection in a diabetic mouse or patient will be successfully controlled or progress to sepsis are not well understood. Neutrophils from diabetic mice without any apparent infections already have functional abnormalities, including decreased phagocytic capacity (44) and chemotactic responses (45). In addition, increased numbers of tissue MCs have been found in clinical diabetes (46). Studies using experimental models also demonstrated that MCs are more abundant in different tissues of diabetic rats compared with related nondiabetic rats (47). Consistent with these findings, we observed that the increase in peritoneal MC numbers in diabetic mice coincided with the severity of sepsis and impaired neutrophil migration during septic peritonitis.

Based on these results, we evaluated the role of MCs in the increased sepsis susceptibility of diabetic mice using several approaches. Pretreatment of diabetic mice with C48/80 induced marked neutrophil migration to the focus of infection, which was associated with decreased bacteremia and an improvement in the survival rate after MS. In agreement, we observed that the high
TNF-α and CXCL-2 levels in the serum of diabetic mice undergoing septic peritonitis were decreased by C48/80 treatment. The improved control of septic peritonitis in C48/80-pretreated diabetic mice could not be attributed to glucose metabolism effects, as the drug did not alter hyperglycemia. C48/80 pretreatment was previously demonstrated to prevent neutrophil migration impairment and systemic mediator release after SS (23). A different study showed that, after MC stabilization using cromolyn sodium, mice were protected from a lethal dose of i.v. Gram-negative bacteria (48). Overall, our results implicate MCs in both neutrophil migration impairment and systemic inflammatory mediator release in response to septic stimulus in diabetic mice.

Chemokines and their receptors play a pivotal role in regulating the activation and migration of leukocytes through the extracellular matrix (49). The importance of CXC-ELR+ chemokines and their receptors for host defense against infection has been demonstrated in various models (50, 51). In humans (52) and experimental sepsis (13), reduced neutrophil chemotaxis and migration were associated with decreased CXCR2 expression on circulating neutrophils. Similarly, we found that CXCR2 expression in neutrophils from diabetic mice undergoing MS expressed high levels of intracellular GRK2, but this expression was diminished after C48/80 pretreatment. Our findings also indicate that the neutrophil migration failure induced by MC degranulation in diabetic mice is dependent on decreased CXCR2 expression mediated by increased intracellular GRK2.

Because we found a large number of MCs in the peritoneal cavity of diabetic mice, we focused our investigation on determining which factors released by MCs enhance diabetic mouse susceptibility to septic peritonitis. One recent study reported that hyperglycemia may trigger a sequence of events leading to an enhancement of basal histidine decarboxylase activity and elevated histamine production after LPS challenge (34). Likewise, serum levels of histamine were higher in diabetic mice than in nondiabetic mice after septic peritonitis induction. Our data suggest that the vast majority of serum histamine is derived from preformed MC granules because C48/80 pretreatment drastically reduced systemic histamine release. In parallel, we observed that diabetic mouse mortality after MS declined after treatment with an H2 antagonist, correlated with neutrophil migration, and re-established CXCR2 expression on these cells. Our research group already reported that the pretreatment with MK886 (LTB4 synthesis inhibitor) of mice given septic peritonitis induced by CLP reduced the neutrophil migration in the peritoneal cavity and survival rate when compared with untreated mice (54). In agreement, other studies using mice lacking LTB4-specific receptor (BLT-1) (55) or treated with the LTB4 receptor antagonist (56) confirmed that LTB4 promotes the neutrophil migration into peritoneal cavity and local chemokine production after CLP. Because histamine causes the inhibition of LTB4 production via H2 (30), and this mediator plays an important role in neutrophil migration and protection to sepsis, we might not exclude that the deleterious effects of histamine are, at least in part, dependent on decreased levels of LTB4 in this experimental model. Taken together, our results suggest that histamine...
Histamine leads to sepsis onset in diabetic mice

Histamine of H1 and H2 did not alter the survival rates in MS diabetic endothelial cells. These studies support our finding that the antagonism expression in endothelial cells is promoted by histamine via H1 chemotaxis (60). On the other hand, increased adhesion molecule antagonist reversed the inhibitory effect of histamine on neutrophil migration. Histamine administration to non-diabetic mice impaired neutrophil migration in a CXCR2-dependent manner. To gain further insight regarding the influence of histamine on CXCR2 regulation, we confirmed that histamine reduced neutrophil chemotaxis and promoted the CXCR2 desensitization associated with GRK2 upregulation. A previous study demonstrated that histamine stimulates the random motility (chemokinesis) and inhibits the directional motility (chemotaxis) of neutrophils in vitro. This study also showed that cAMP levels in neutrophils are significantly increased after histamine incubation and decreased after treatment with an H2 antagonist (64). Complementing these studies, we demonstrated an additional effect of histamine in neutrophil migration by induction of CXCR2 receptor desensitization perhaps via GRK2 upregulation. In support, another study also found that histamine-deficient mice control Escherichia coli infection better than wild-type mice, which was related to high leukocyte migration (43). Also, importantly, histamine is induced during acute M. tuberculosis infection and may dampen protective immunity by augmenting the inflammatory response associated with this disease (65).

MCs have been proposed to enhance survival by augmenting intracellular neutrophil killing through either IL-6 production (66) or through the action of MC proteases (67). In general, our findings support that activation of local MCs is crucial for host defense during bacterial infection because nondiabetic W/Wv mice had more sepsis mortality than WB/B6 nondiabetic mice. In contrast, we showed that diabetic W/Wv mice had lower sepsis mortality than WB/B6 diabetic mice. In agreement, other studies have shown that systemic MC activation exacerbates mortality during SS (24, 68). Although MCs may induce neutrophil recruitment and activation, and could have a protective role during acute local infection, these effects can be overcome and ultimately become detrimental during systemic and severe infections. In addition, along with improved survival, diabetic W/Wv mice undergoing MS had lower numbers of bacteria in the blood and increased neutrophil migration into the peritoneal cavity that were associated with decreased CXCR2 internalization, indicating that MC deficiency confers resistance to septic peritonitis in diabetic mice.

NOD mice spontaneously develop a form of diabetes that closely resembles the human disease (69). Accordingly, NOD mice were more susceptible to MS than prediabetic mice, with 50 and 25% mortality rates, respectively. Septic peritonitis was exacerbated in NOD mice when compared with prediabetic mice because these mice were unable to clear the polymicrobial infection, which then progresses to bacteremia. These effects correlated with increased MC numbers, increased serum histamine levels, impaired neutrophil migration, and downregulation of CXCR2 expression on blood neutrophils. H2 pretreatment of NOD mice promoted neutrophil chemotaxis and migration associated with maintenance of CXCR2 expression and, consequently, a better control of bacterial infection.

In conclusion, this study shows that MC degranulation induces neutrophil migration failure through CXCR2 receptor desensitization by a mechanism that is dependent on augmented GRK2 expression and that ultimately results in sepsis development. In addition, to our knowledge, we provide the first evidence that MCs appear to exert these harmful effects through the systemic release of histamine. Taken together, our results suggest that inhibition of histamine-mediated signaling through H2 antagonism might confer protection against sepsis onset in a diabetic host.

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Disclosures

The authors have no financial conflicts of interest.
References


Supplementary Material

Supplementary Fig. 1. Experimental design and clinical parameters after diabetes induction. Diabetes was induced with a single administration i.v. of alloxan at a dosage of 50 mg/kg in 100 μl of saline (vehicle). Compound 48/80 (C48/80) was administered two times per day from day 6 to day 9. At day 10, diabetic and non-diabetic mice underwent mild sepsis (MS) or severe sepsis (SS) by CLP, and sham-operated mice were used as a control. Non-diabetic mice were pre- and post-treated s.c. with histamine, and diabetic mice were pre- and post-treated i.p. with histamine receptor antagonists (H1-H4) 1 hour before CLP and every 24 hours afterwards for a total of 5 days to determine survival rate. Mice were killed at 2, 4, or 6 hours after septic stimulus or sham surgery (A). Blood glucose levels were measured 10 days after administration of alloxan (Diabetic mice) and compound 48/80 pretreatment (Diabetic + C48/80) (B). The non-diabetic mice were inoculated with 100 μL of saline. The body weight variation is expressed as the mean ± SEM (n=8) (C). Mast cell numbers were quantified in the peritoneal cavity using mast cell-specific markers (CD117 and FceRI) in naïve non-diabetic or diabetic mice 5 days after alloxan administration (D). *p≤0.05 compared to the non-diabetic group.

Supplementary Figure 2. MC granule depletion attenuates systemic inflammation in diabetic mice during septic peritonitis and induces protection to severe sepsis in non-diabetic mice. Blood was collected from sham-operated, non-diabetic, diabetic mice treated with saline or C48/80 undergoing mild sepsis (MS) and non-diabetic mice undergoing severe sepsis (SS). The leukocyte trapped in lungs was assessed by myeloperoxidase (MPO) assay 6 hours after CLP surgery (A). TNF-α (B) and CXCL-2 (C) concentrations in the serum were assessed by ELISA. Non-diabetic mice were pretreated with saline or C48/80 before induction of mild sepsis.
C48/80 after induction of mild sepsis (MS) or severe sepsis (SS) were evaluated daily for 5 days after CLP surgery (D). GRK2 expression was evaluated by flow cytometry in blood neutrophils (E). The results are expressed as the mean ± SEM (n=5-7). *p≤0.05 compared to the non-diabetic sham-operated group; #p≤0.05 compared to the non-diabetic MS group, †p≤0.05 compared to the non-diabetic SS group; &p≤0.05 compared to the diabetic MS group.

Supplementary Figure 3. Histamine inhibits the neutrophil chemotactic activity in diabetic mice and induces intracellular GRK2 expression. Blood neutrophils from sham-operated, non-diabetic (ND), and diabetic mice pretreated with either saline or an H2 antagonist were obtained 2 hours after mild sepsis (MS) induction. The chemotactic activity of blood neutrophils toward fMLP (10⁻⁷ M) or CXCL-2 (30 ng/ml) was determined in Boyden chambers (A). In another set of experiments, bone marrow neutrophils were incubated with histamine (10 μM) for 1 hour before assessing GRK2 expression. Neutrophils were also preincubated with an H2 antagonist for 1 hour before histamine incubation. GRK2 expression was evaluated by flow cytometry (B) or immunofluorescence (C). Blue staining by DAPI represents neutrophil nuclei, and red staining represents GRK2 expression in the neutrophil cytoplasm. An overlay of nuclei and GRK2 expression shows the qualitative analysis from several experimental groups. *p≤0.05 compared to the non-diabetic sham-operated group or histamine incubation; #p≤0.05 compared to the non-diabetic MS group; &p≤0.05 compared to the diabetic MS group.

Supplementary Figure 4. Histamine dampens CXCR2 expression on neutrophils from NOD mice. Blood neutrophils from sham-operated, pre-diabetic or NOD mice pretreated with either saline or an H2 antagonist were obtained 2 hours after mild sepsis (MS) induction. Quantitative
analysis of the mean intensity of fluorescence (MIF) for CXCR2 in blood cells with high expression of GR-1 (neutrophils) was evaluated by flow cytometry (A). The chemotactic activity of blood neutrophils toward CXCL-2 (30 ng/ml) or RPMI 1640 (negative control) was determined in Boyden chambers (B). The results are expressed as the mean ± SEM (n=4-6).

*p≤0.05 compared to the sham-operated pre-diabetic NOD group or after RPMI 1640 incubation; #p≤0.05 compared to the pre-diabetic NOD MS group; ∞p≤0.05 compared to the NOD MS group.
A. Neutrophil chemotaxis (cells/field)

- fMLP (10^{-7} M)
- CXCL-2 (30ng/ml)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Neutrophil Chemotaxis</th>
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<tbody>
<tr>
<td>Sham</td>
<td>ND</td>
</tr>
<tr>
<td>MS Diabetic</td>
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<tr>
<td>MS H_{2}</td>
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<tr>
<td>Sham Diabetic</td>
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B. GRK2 % (Relative to RPMI)

- Histamine (μM)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>GRK2 %</th>
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<tbody>
<tr>
<td>10</td>
<td>120</td>
</tr>
<tr>
<td>10 H_{2}</td>
<td>100</td>
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C. Imaging

- RPMI
- Histamine
- H_{2}

- DAPI
- GRK2
- MERGE