α1-Acid Glycoprotein Decreases Neutrophil Migration and Increases Susceptibility to Sepsis in Diabetic Mice

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The mechanisms underlying immune deficiency in diabetes are largely unknown. In the present study, we demonstrate that diabetic mice are highly susceptible to polymicrobial sepsis due to reduction in rolling, adhesion, and migration of leukocytes to the focus of infection. In addition, after sepsis induction, CXCR2 was strongly downregulated in neutrophils from diabetic mice compared with nondiabetic mice. Furthermore, CXCR2 downregulation was associated with increased G-protein–coupled receptor kinase 2 (GRK2) expression in these cells. Different from nondiabetic mice, diabetic animals submitted to mild sepsis displayed a significant augment in α1-acid glycoprotein (AGP) hepatic mRNA expression and serum protein levels. Administration of AGP in nondiabetic mice subjected to mild sepsis inhibited the neutrophil migration to the focus of infection, as well as induced L-selectin shedding and rise in CD11b of blood neutrophils. Insulin treatment of diabetic mice reduced mortality rate, prevented the failure of neutrophil migration, impaired GRK2-mediated CXCR2 downregulation, and decreased the generation of AGP. Finally, administration of AGP abolished the effect of insulin treatment in diabetic mice. Together, these data suggest that AGP may be involved in reduction of neutrophil migration and increased susceptibility to sepsis in diabetic mice. *Diabetes* 61:1584–1591, 2012

Accounting for up to an estimated 22% of deaths in diabetic individuals, infection is a common and severe complication of diabetes, as well as a well-recognized cause of mortality (1). In addition, it has been proven by murine experimental models that diabetic mice are at higher risk for a variety of bacterial infections (2–5). Despite these considerations, the mechanism behind immune deficiency in diabetes is largely unknown.

In this context, deficiencies in neutrophil functions have been described in diabetic host and include the following: adhesion and migration to the site of inflammation (6), chemotaxis (7), phagocytosis capacity (8), and microbicidal activity (9). The major consequence of the high susceptibility to infections in diabetic patients is the development of sepsis and, ultimately, septic shock (10). Our group has

previously shown that failure of neutrophil migration to infection sites is associated with a poor outcome in experimental sepsis (11). Furthermore, neutrophils obtained from nonsurvival septic patients displayed a significant reduction in chemotactic response compared with neutrophils from healthy subjects or surviving septic patients (12).

The mechanisms involved in the reduction of neutrophil migration during sepsis are not completely understood. However, excessive levels of circulating cytokines/chemokines and LPS induce CXCR2 chemokine receptor internalization, as well as upregulation of G-protein–coupled receptor kinase 2 (GRK2) expression in neutrophils (13,14). These events culminate in decreased endothelium-leukocyte interactions and diminished neutrophil chemotactic responses. Additionally, α1-acid glycoprotein (AGP) is involved in the failure of neutrophil migration after human sepsis (15). In this context, changes in glycosylation and higher serum levels of AGP were found in patients with diabetes (16,17).

In the current study, we demonstrate that diabetic mice have a reduction of neutrophil migration to the focus of infection after polymicrobial sepsis. Moreover, we have provided evidence for the first time that in diabetic mice, these events are mediated by CXCR2 downregulation and upregulation of GRK2 in neutrophils. Interestingly, our results also suggest that these events may be mediated by AGP during diabetes development.

RESEARCH DESIGN AND METHODS

Care and treatment of animals was based on the Guide for the Care and Use of Laboratory Animals (18), and research was approved by the Animal Research Ethics Committee of the School of Medicine Ribeirão Preto. Balb/C and C57BL/6 (8–10 weeks old) mice were housed in an adequate animal room at 23–25°C, with free access to water and food.

Diabetes induction. Diabetes was induced by a single intravenous injection of 50 mg/kg body wt alloxan (Sigma) in saline (100 μL). The control group received an intravenous injection of saline (100 μL). Diabetic mice with a blood glucose level ≥300 mg/dL 3 days after alloxan treatment were used. In the fifth day, alloxan-treated animals were treated subcutaneously daily with saline or insulin for 5 days. One dose of bovine insulin (Sigma) was administered in the morning, and one dose of insulin glargine (long-acting insulin; sanofi-aventis) was administered at 5 P.M. Both were administered at a dose of 1 UI for each 200 mg/dL blood glucose. Blood samples were collected from the tail vein to measure blood glucose levels by a glucometer (Precision Xtra; Abbott). Ten days after alloxan treatment, mice were submitted to sham operation (SH), mild sepsis (MS), or severe sepsis (SS) by the cecum ligation and puncture (CLP) model and several parameters were analyzed. In another set of experiments, at day 10 after alloxan treatment, the mice were used for neutrophil migration assay in vivo or for blood neutrophil chemotaxis assay in vitro.

Sepsis model. Sepsis was induced by CLP (19). Two punctures were made in the cecum using a 21-gauge needle to induce MS or using an 18-gauge needle to induce SS. SH animals were submitted to laparotomy without cecal puncture.

Neutrophil migration to peritoneal cavity and lung. Neutrophil migration to peritoneal cavity or to lung cavity was evaluated 6 h after sepsis induction or 6 h after intraperitoneal injection of macrophage inflammatory protein (MIP)-2 (chemokine [C-X-C motif] ligand (CXCL)-2, 3–30 ng/mice; R&D Systems).

Tissue myeloperoxidase assay. Extent of leukocyte accumulation in the lung was measured 6 h after sepsis by myeloperoxidase activity as previously described (20).

Bacterial counts. Bacterial count was determined 6 h after sepsis in peritoneal cavity and blood as previously described (21).

Determination of serum levels of alanine aminotransferase and aspartate aminotransferase. Using colorimetric assay kits, serum alanine aminotransferase and aspartate aminotransferase (Labtest Diagnóstica, Lagoa Santa, Brazil) levels were estimated 6 h after sepsis.

Determination of serum levels of insulin. Using an ELISA assay kit (Millipore), serum insulin levels were estimated 6 h after sepsis.

Leukocyte rolling/adhesion to mesenteric microcirculation. Briefly, mice were anesthetized with tribromoethanol (250 mg/kg i.v.) and mesenteric tissue was withdrawn for intravital microscopic examination 3 h after surgery as previously described (20).

Neutrophil isolation and chemotaxis assay. Blood neutrophils were isolated by Percoll gradient as previously described (14). Next, neutrophil chemotaxis assay (10^6 cells/mL in RPMI-1640, 0.01% BSA, 97% of viable cells, assessed by trypan blue) was performed using a 48-well modified Boyden chamber (Neuro Probe) in response to RPMI-1640 or MIP-2 (10 or 30 ng/mL).

Measurement of seric AGP. AGP was measured in serum by an immunoturbidimetric method using a kit manufactured by Quibasa Química Básica (Belo Horizonte, Minas Gerais, Brazil).

Detection of cytokines by ELISA. MIP-2, keratinocyte-derived chemokine (KC), and interleukin (IL)-6 levels were detected in serum and peritoneal cavity by ELISA 6 h after surgery.

Flow cytometry. Mouse blood samples were stained with rat anti-mouse R-phycoethrin-CD62L (MEL-14), fluorescein isothiocyanate–CD11b (M1/70), Peridinin-chlorophyll proteins (PerCP)-Cy5.5-GR1 (Ly-6G) (BD Bioscience), and R-phycoethrin-CXCR2 (R&D Systems) monoclonal antibodies or appropriate isotype controls. After staining, cells were fixed, washed, and analyzed using a flow cytometer (FACSort; Becton Dickinson).

Immunofluorescence of GRK2. Neutrophils were affixed on glass slides and then incubated with rabbit anti-mouse GRK2 antibody or isotype control (Santa Cruz Biotechnology). Next, Alexa-Fluor 594–conjugated goat anti-rabbit IgG antibody (Invitrogen) was added. Cells were incubated with DAPI (Invitrogen) to stain the cell nucleus. Microscopic analysis of fluorescent images was performed using an Olympus BX-50 epifluorescence microscope.

AGP real-time PCR. Four hours after surgery, mice were killed and the liver was collected. Total RNA was extracted with TRIzol reagent (Invitrogen) using instructions from the manufacturer. Reverse transcription of total RNA to cDNA was carried out with reverse transcription reaction (Superscript II; Gibco Life Technologies). Real-time PCR quantitative mRNA analysis was performed in an ABI Prism 7500 Sequence Detection System using the SYBR–green fluorescence system (Applied Biosystems) as indicated by the manufacturer. Data were analyzed with the comparative cycle threshold method. Primer pairs for mouse β-actin and AGP were as follows: AGP forward, 5′-CACAGCAAATAGTTCAACCA-3; AGP reverse, 5′-CAAAGGTTTCTACTCCTCCT-3′; β-actin forward, 5′-AGCT-GCGTTTTACACCCTTT-3′; and β-actin reverse, 5′-AAGCCATGCCAATGTTGTCT-3′. All samples were submitted to dissociation characteristics of double-stranded DNA during heating (melting curve analysis). To validate and standardize the method, we used positive or negative known control samples for both AGP and β-actin genes.

Statistical analysis. Data are reported as means ± SEM (except for survival curves) and are representative of two or three independent experiments with five mice in each group. Differences between two unpaired groups were compared by Student t test. The mean differences between three or more groups within an experiment were compared by ANOVA. When significant differences were identified, individual comparisons were tested subsequently with Bonferroni t test. Statistical significance was set at $P < 0.05$. Survival rates ($n = 10$–12) were expressed as percentages, and a log-rank test (χ^2 test) was used to examine differences between survival curves.

RESULTS

Diabetic mice died after MS induction. Alloxan-induced diabetes has commonly been used as an animal model of insulin-dependent diabetes (22–24). Compared with those in control nondiabetic mice, blood glucose levels were found to be elevated in alloxan-treated mice (Fig. 1A). Moreover, body weight gain was observed in nondiabetic mice during the observation period (10 days, 2 ± 0.2 g), whereas diabetic mice presented significant weight loss (−3.2 ± 1.4 g). Insulin-replacement therapy reversed the state of metabolic catabolism, as observed by decreased blood glucose levels (Fig. 1A) and recovery of weight gain: increase of 0.25 ± 0.31 g. Taken together, these data confirmed the presence of severe diabetes in this mouse model.

We next investigated the influence of alloxan treatment on sepsis outcome. Diabetic mice submitted to MS (10 days after alloxan treatment) showed 100% mortality after 4 days, whereas ~85% of control mice survived until 7 days after surgery (Fig. 1B). Induction of MS 5 days after alloxan treatment also decreases survival rate compared with nondiabetic mice (Supplementary Fig. 1A). All SH control and diabetic mice survived during the observation period (data not show). As previously demonstrated (21), all nondiabetic mice submitted to SS died during the 3 day period after sepsis induction. As expected, diabetic mice exposed to SS died faster than nondiabetic mice (Supplementary Fig. 1B). Similar effects on blood glucose, weight loss, and mortality were also observed in a different mouse strain (Supplementary Fig. 2A–C). In accordance with survival data, diabetic mice subjected to MS or control mice subjected to SS failed to control the infection, as demonstrated by the higher number of colony-forming units (CFUs) in peritoneal cavity and blood compared with MS control mice (Fig. 1C and D).

Deficiencies in neutrophil migration have been described in experimental diabetic models (7). Here, we observed that diabetic animals with MS displayed a significant impairment of neutrophil migration into the peritoneal cavity, which was similar to that observed in nondiabetic mice subjected to SS (Fig. 1E).

The reduction of neutrophil migration to the infection site in diabetic mice was not a consequence of low concentration of neutrophil chemotactic cytokine in the focus of infection. The levels of MIP-2 (CXCL2) in the peritoneal cavities were 77.2 ± 18.0 pg/mL (SH), 423.4 ± 132.6 pg/mL (MS), and 1,893 ± 85.9 pg/mL (SS) for control mice ($n = 5$) and 99.6 ± 32.0 pg/mL (SH) and 2,017 ± 51.6 pg/mL (MS) for diabetic mice ($n = 5$). The peritoneal levels of tumor necrosis factor-α were 28.0 ± 3.0 pg/mL (SH), 119.3 ± 28.2 pg/mL (MS), and 505.8 ± 87.5 pg/mL (SS) for control mice ($n = 5$) and 25 ± 9.5 pg/mL (SH) and 310.8 ± 45.4 pg/mL (MS) for diabetic mice.

The elevated serum glucose levels and/or low levels of insulin associated with diabetes may be responsible for disturbance in the host immune response (25). Therefore, we subjected diabetic mice to insulin therapy followed by CLP. Expectedly, insulin treatment significantly prevented high mortality rate of diabetic MS mice (Fig. 1B), decreased the bacterial load in the focus of infection and blood (Fig. 1C and D), and improved neutrophil migration toward the infection site (Fig. 1E).

To investigate whether the observed effects of insulin treatment in diabetic septic mice were specific to a diabetes-associated phenomenon, we also evaluated the outcome of control nondiabetic mice exposed to insulin during sepsis. Nondiabetic animals subjected to SH, MS, or SS did not present significant changes in serum levels of insulin 6 h after surgery (Supplementary Fig. 3A). As expected, alloxan-treated mice displayed reduced serum insulin amounts, which increased during treatment.

After 2 h postsepsis, all animal groups displayed similar blood glucose concentration, indicating that the anesthesia (ketamine/xilasine) rather than sepsis induced hyperglycemia, as previously published by Saha et al. (26). After the observed hyperglycemia, nondiabetic septic, but not sham or naïve animals, displayed low levels of blood glucose (Supplementary Fig. 3B and data not shown). In contrast with the observed effect on diabetic septic mice (Fig. 1B), insulin administration (2 or 1 UI s.c. 30 min after surgery) in nondiabetic septic mice promoted significant mortality rates (Supplementary Fig. 3C–F), which could be
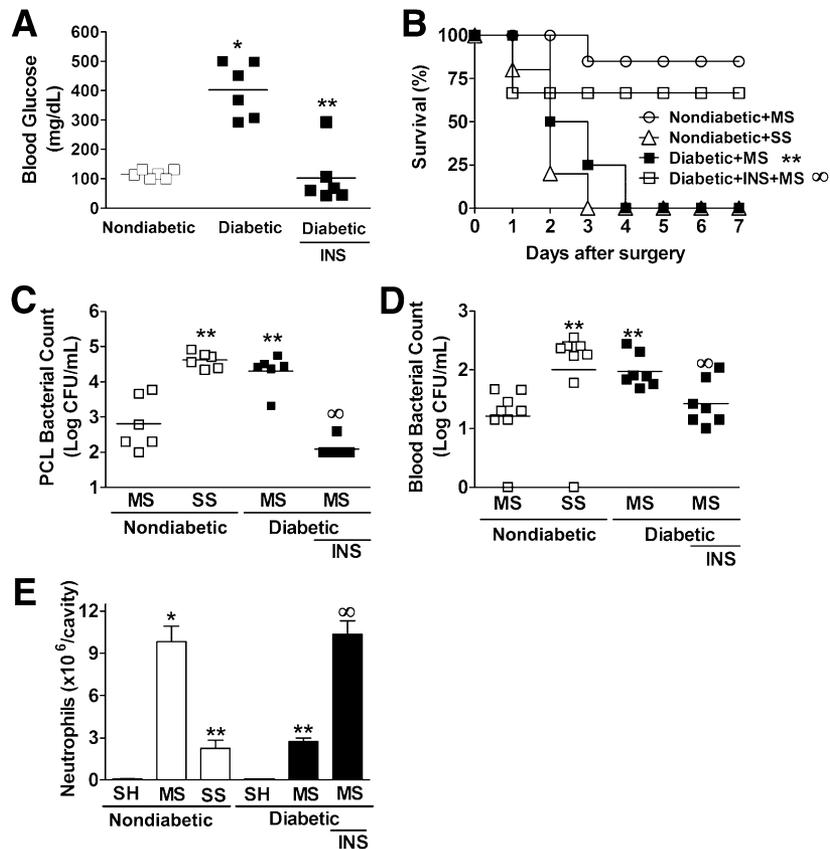
FIG. 1. Mice subjected to alloxan-induced insulin-dependent diabetes died after MS induction. *A*: Blood glucose levels were measured 10 days after administration of alloxan (diabetic group), alloxan plus insulin treatment (diabetic + INS group), or saline (nondiabetic group); *$P < 0.01$ compared with nondiabetic group; **$P < 0.01$ compared with diabetic group. Ten days after alloxan or saline administration, nondiabetic or diabetic mice were subjected to SH, MS, or SS, and survival rate (*B*), bacterial load in peritoneal cavity lavage (PCL) (*C*), or blood (*D*) or neutrophil migration to peritoneal cavity (*E*) were evaluated 6 h after surgery. The survival rate was determined daily up to 7 days after surgery, and other parameters were determined 6 h after surgery. *$P < 0.01$ compared with SH nondiabetic group; **$P < 0.01$ compared with MS nondiabetic group; ∞$P < 0.05$ compared with MS diabetic group. Diabetic mice were treated with insulin for 5 days (diabetic + INS group). (See RESEARCH DESIGN AND METHODS.) Values are means ± SEM (*n* = 5) and are representative of three independent experiments.

explained by exacerbation of hypoglycemic state, i.e., treatment of already hypoglycemic mice with insulin (27). Together, these data suggest that specific defects associated with diabetes enhance sepsis sensitivity, which can be rescued by insulin treatment.

Systemic inflammation in diabetic septic mice was prevented by insulin treatment. High levels of systemic inflammatory cytokines, as well as leukocyte sequestration into remote tissues of infection site, were correlated with the severity of sepsis (13). Therefore, we determined leukocyte sequestration into the lung and serum levels of cytokines 6 h after CLP. MS diabetic mice had significantly higher serum concentrations of MIP-2 and KC than found in resistant MS nondiabetic mice (Fig. 2*A* and *B*). Serum levels of MIP-2, KC, and IL-6 in SS nondiabetic mice were similar to the levels found in MS diabetic mice. Notably, insulin treatment in MS diabetic mice significantly inhibited the elevation of MIP-2, KC, and IL-6 serum levels.

Leukocyte sequestration (Fig. 2*D*) in the lungs and neutrophil migration to broncoalveolar cavity (Supplementary Fig. 4) of MS diabetic mice were significantly higher than in MS nondiabetic mice. Insulin-treated diabetic mice presented significantly reduced leukocyte sequestration and neutrophil migration in lungs. In addition to systemic inflammatory response, diabetic mice subjected to MS presented significantly higher serum levels of aspartate aminotransferase and alanine aminotransferase (biochemical

markers of liver injury) compared with nondiabetic MS mice. Insulin treatment was able to prevent the increased alanine aminotransferase and aspartate aminotransferase serum levels (Supplementary Fig. 5).

Diabetic mice exhibit decreased leukocyte rolling/adherence to mesenteric microcirculation and decreased neutrophil chemotaxis response. Since neutrophil migration depends on leukocyte-endothelium interactions and chemotaxis (28), we examined the effect of diabetes on these parameters. Diabetic MS mice and nondiabetic SS mice displayed a significant decrease in leukocyte rolling and adhesion compared with nondiabetic MS animals (Fig. 3*A* and *B*). Importantly, insulin treatment of diabetic mice significantly prevented the reduction of leukocyte rolling and adhesion after CLP surgery (Fig. 3*A* and *B*).

As shown in Fig. 3*C*, MS induction in diabetic mice or SS induction in nondiabetic mice resulted in significant inhibition of MIP-2–induced chemotaxis of circulating neutrophils compared with neutrophils from MS nondiabetic mice. Insulin treatment also restored the neutrophil chemotaxis response to MIP-2 in MS diabetic mice. Interestingly, blood neutrophils from diabetic mice without sepsis presented a significant decline in neutrophil chemotaxis response (Supplementary Fig. 6*A*). In vivo, intraperitoneal administration of MIP-2 also induced a lower neutrophil migration in diabetic mice compared with cell migration in nondiabetic mice (Supplementary Fig. 6*B*).
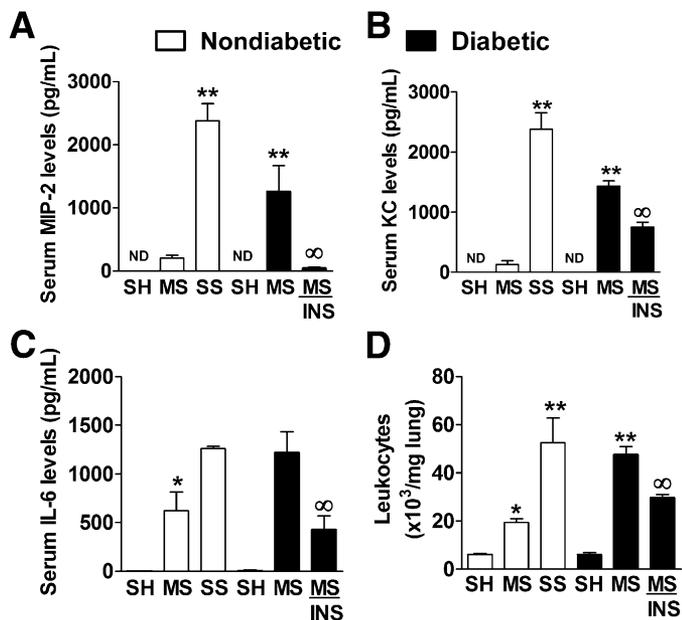
FIG. 2. Systemic inflammation in diabetic mice during MS was prevented by insulin treatment. Ten days after alloxan or saline administration, nondiabetic or diabetic mice were subjected to SH, MS, or SS, and 6 h after surgery, seric levels of MIP-2 (*A*), IL-6 (*B*), and KC (*C*) or leukocyte infiltration (*D*) in lung was determined. *$P < 0.01$ compared with SH nondiabetic group; **$P < 0.05$ compared with MS nondiabetic group; $\infty P < 0.05$ compared with MS diabetic group. Diabetic mice were treated with insulin (INS) for 5 days. (See RESEARCH DESIGN AND METHODS.) Values are means ± SEM ($n = 5$) and are representative of three independent experiments.

FIG. 3. Diabetic mice exhibit decreased leukocyte rolling/adherence to mesenteric microcirculation and decreased neutrophil chemotaxis response. Ten days after alloxan or saline administration, nondiabetic or diabetic mice were subjected to SH, MS, or SS, and 3 h after surgery the leukocyte rolling (*A*) and adhesion (*B*) were determined in the microcirculation of mesenteric tissue. *C*: Six hours after surgery, blood neutrophils were isolated and subjected to chemotaxis response to MIP-2 (30 ng/mL) in a Boyden chamber assay. *$P < 0.01$ compared with SH nondiabetic group; **$P < 0.05$ compared with MS nondiabetic group; $\infty P < 0.05$ compared with MS diabetic group. Diabetic mice were treated with insulin (INS) for 5 days. (See RESEARCH DESIGN AND METHODS.) Values are means ± SEM ($n = 5$) and are representative of three independent experiments.

High serum levels of AGP are correlated with inhibition of neutrophil migration in diabetic mice after sepsis.

High levels of AGP have been reported in human diabetes (16,17), and this acute-phase protein is involved in neutrophil migration failure during sepsis (15). Figure 4*A* shows that serum levels of AGP were significantly increased 6 h after surgery in SH and MS diabetic mice compared with those in SH and MS control mice. Moreover, serum levels of AGP were comparable in MS diabetic mice and SS nondiabetic mice. Similarly, expression of AGP mRNA measured by real-time quantitative PCR in liver tissues was significantly higher in SH or MS diabetic mice than in SH or MS nondiabetic mice, respectively. Treatment of diabetic mice with insulin resulted in significant inhibition of AGP mRNA expression in the liver (Fig. 4*B*). AGP serum levels were negatively correlated with neutrophil migration (Fig. 4*C*) and positively correlated with serum levels of alanine aminotransferase and aspartate aminotransferase (Supplementary Fig. 5*C* and *D*, respectively). Confirming the inhibitory role of AGP in neutrophil migration, we observed intravenous administration of AGP (50 µg/mice) 30 min before induction of MS inhibits neutrophil migration to the focus of infection (Fig. 4*D*).

Adhesion molecules such as CD11b and CD62L are important for rolling and adhesion as well as useful markers of neutrophil activation (29). Thus, expression of L-selectin (CD62L) and CD11b in neutrophils was evaluated. Neutrophils obtained from SS nondiabetic mice or from diabetic mice subjected to MS presented a significant reduction in CD62L expression (Fig. 4*F*) and upregulation of CD11b (Fig. 4*G*) compared with MS nondiabetic mice. Insulin-treated diabetic mice impaired exacerbation of neutrophil L-selectin shedding and upregulation of CD11b after sepsis.

Remarkably, when AGP was administered to insulin-treated MS diabetic mice, the effect of insulin in preventing the reduction of neutrophil migration as well as L-selectin shedding and rise of CD11b in blood neutrophils was abolished (Fig. 4*D*, *F*, and *G*, respectively). Figure 4*E* shows representative histograms of CD62L and CD11b expression in blood neutrophils (cells with high expression of GR-1).

Diabetic mice display reduced neutrophil chemotaxis to MIP-2, CXCR2 internalization, and GRK2 upregulation after MS induction.

Since CXCR2 expression in leukocytes is necessary for neutrophil migration to infection site (30) and chemotaxis induced by MIP-2 (14), expression of CXCR2 in neutrophils was evaluated. Neutrophil CXCR2 expression in MS diabetic mice or SS nondiabetic mice was significantly decreased compared with neutrophils from MS nondiabetic mice. Treatment of diabetic mice with insulin prevented CXCR2 internalization in neutrophils after MS induction (Fig. 5*A*). Administration of AGP to MS nondiabetic
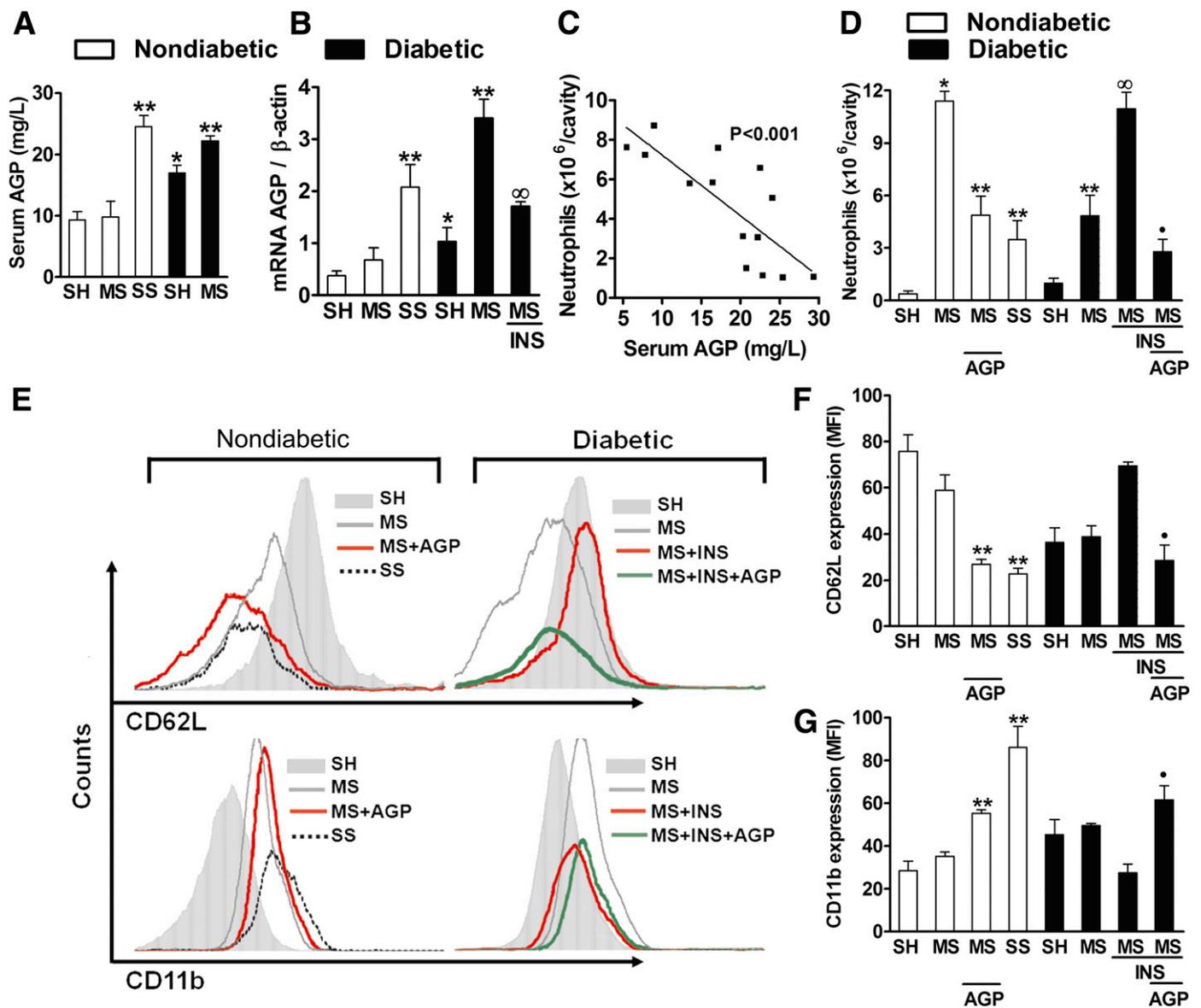
FIG. 4. High serum levels of AGP are correlated with inhibition of neutrophil migration in diabetic mice after sepsis. Ten days after alloxan or saline administration, nondiabetic or diabetic mice were subjected to SH, MS, or SS, and 6 h after surgery seric levels of AGP (A) and 4 h after surgery hepatic rate of mRNA AGP/β-actin (B) were evaluated. C: Correlation between number of neutrophils in the peritoneal cavity (y-axis) and seric levels of AGP (x-axis) 6 h after surgery. Thirty minutes before surgery, saline or AGP (50 μg/mice) was administered intravenously, and neutrophil migration to peritoneal cavity (D) or expression of CD62L (F) or CD11b (G) in blood cells with high expression of GR-1 (neutrophils) was determined 6 h after surgery. E: Representative histograms of fluorescence intensity (means of fluorescence intensity [MFI]) of CD62L and CD11b in blood cell with high expression of GR-1 measured by FACSort flow cytometry. Values are means ± SEM ($n = 5$) and are representative of three independent experiments. *$P < 0.01$ compared with SH nondiabetic group; **$P < 0.05$ compared with MS nondiabetic group; ∞$P < 0.05$ compared with MS diabetic group; ●$P < 0.05$ compared with MS diabetic plus insulin (INS) group.

or to insulin-treated MS diabetic mice reduced CXCR2 expression in blood neutrophils. Figure 5A shows the representative histograms of CXCR2 expression in blood neutrophils (cells with high expression of GR-1).

High expression of GRK2 has been described as a key modulator of CXCR2 receptor desensitization (14,31). Therefore, we examined by immunofluorescence microscopy whether diabetes development modulates the expression of GRK2 in neutrophils after sepsis. Significant upregulation of intracellular GRK2 protein expression was found in SS nondiabetic mice and MS diabetic mice compared with neutrophils from MS nondiabetic or SH diabetic mice. Insulin treatment prevented the GRK2 increase observed in the MS diabetic mice (Fig. 5B and C).

Administration of AGP to MS diabetic mice treated with insulin increased GRK2 expression in blood neutrophils.

DISCUSSION

Using the alloxan model of diabetes followed by polymicrobial infection, we demonstrated that diabetic mice with poor metabolic control are highly susceptible to sepsis. After MS induction, diabetic mice exhibited a reduction in rolling, adhesion, chemotaxis, and migration of leukocytes. As a consequence, these mice had bacterial spreading, systemic inflammatory response, and 100% mortality. Blood neutrophils from diabetic mice subjected to MS expressed high levels of GRK2 and downregulation of CXCR2
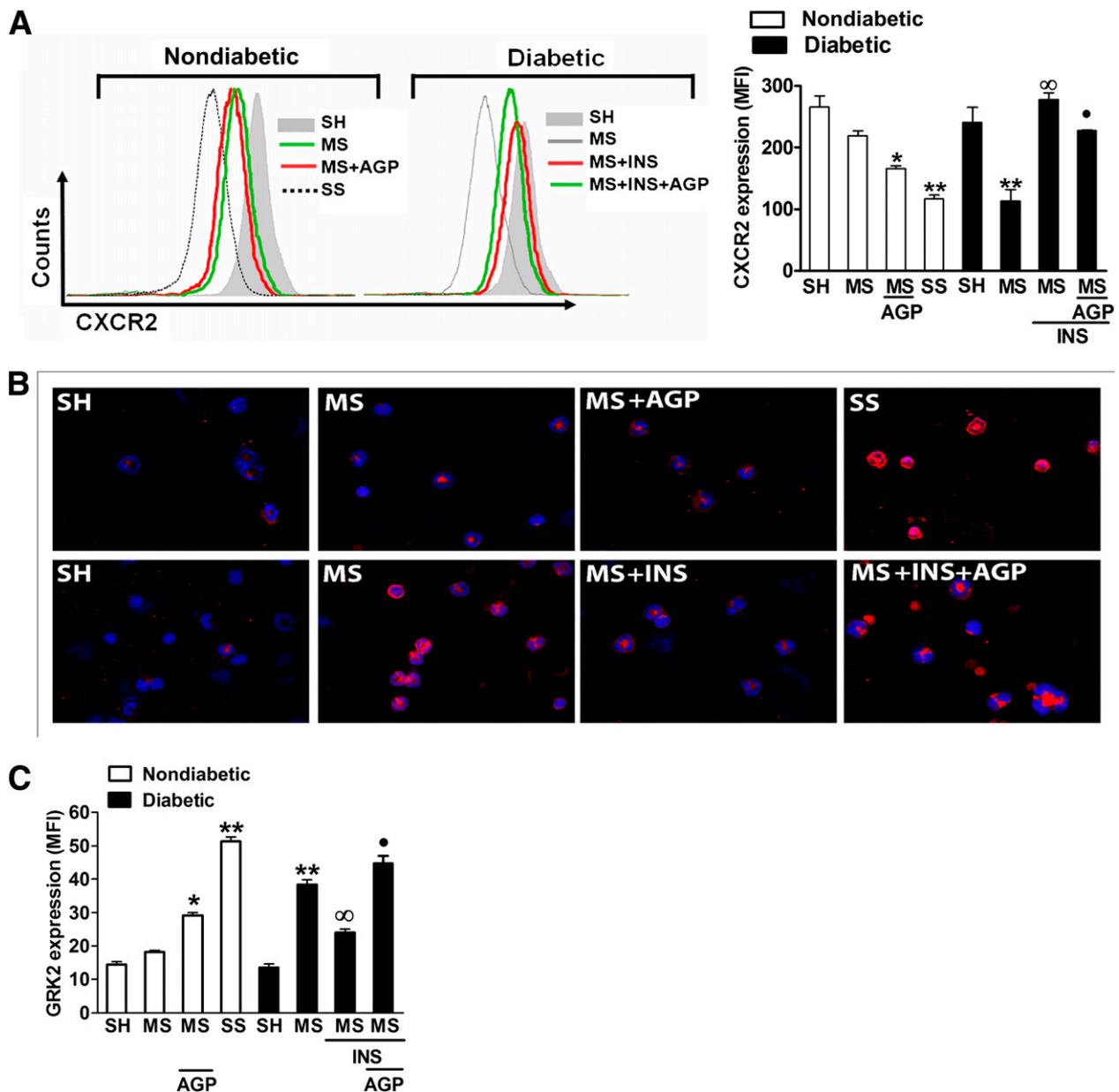
FIG. 5. Diabetic mice display reduced CXCR2 expression and GRK2 upregulation after MS. Ten days after alloxan or saline administration, nondiabetic or diabetic mice were treated with saline or AGP (50 μg/mice) intravenously and mice were subjected to SH, MS, or SS. Six hours after surgery, CXCR2 expression in blood cells with high expression of GR-1 (neutrophils) was evaluated by flow cytometry. A: Representative histograms of fluorescence intensity (means of fluorescence intensity [MFI]) of CXCR2 and quantification of expression (means ± SEM). *P < 0.01 compared with SH nondiabetic group; **P < 0.05 compared with MS nondiabetic group; ∞P < 0.05 compared with MS diabetic group; ●P < 0.05 compared with MS diabetic plus insulin (INS) group. B: Overlay of nuclei and GRK2 expression in blood neutrophils from different experimental groups. Blue staining by DAPI represents neutrophil nuclei, and red staining represents GRK2 expression in neutrophils (×400). C: Semiquantitative analyses of GRK2 expression showed the means of fluorescence intensity. The number of animals per group was five. Three slices per animal were used, and the means of fluorescence intensity of at least 200 neutrophils per slice was determined. The experiment was repeated three times. Values are means ± SEM. (A high-quality digital representation of this figure is available in the online issue.)

expression. Interestingly, the immunosuppression found in diabetic mice appears be mediated by AGP, an acute-phase protein.

Neutrophils are essential for bacterial infection eradication (13,28,32). For diabetes, several studies have demonstrated a defect in neutrophil migration/chemotaxis in human and experimental models (33,34). Extending these findings, we observed that diabetic mice subjected to MS exhibited reduced neutrophil migration to the focus of infection, resulting in bacteremia, systemic inflammatory response, and 100% mortality. The levels of tumor necrosis factor-α and

MIP-2 in the peritoneal lavage of diabetic mice were higher than in nondiabetic mice subjected to the same septic stimulus, suggesting that reduction of neutrophil recruitment is not due to the absence of inflammatory mediators.

Next, we investigated the mechanism by which diabetic mice display reduction of neutrophil recruitment toward the infection site. We demonstrated that diabetic mice subjected to MS had a significant decrease in leukocyte rolling and adhesion in postcapillary venules of mesentery. Furthermore, we observed that diabetic MS mice presented impaired intracellular adhesion molecule-1 mesenteric endothelium

expression compared with nondiabetic MS mice (data not shown). Changes in adhesion molecule expression also could contribute to the impairment of rolling and adhesion of leukocytes to endothelial cells in diabetes (28). Nevertheless, the role of adhesion molecules such as intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 in the sensitivity to MS remains to be elucidated. Our results are in accordance with those of Fortes et al. (35), which showed reduced leukocyte rolling on venular endothelium of the internal spermatic fascia in diabetic rats.

The inappropriate activation of leuckocytes in circulation induced the shedding of L-selectin and upregulation of CD11b, which could decrease the interactions of these cells with the endothelium (36). Our results demonstrated that neutrophils from diabetic mice had a significant CD62L shedding and upregulation of CD11b compared with control mice. In accordance, a significant increase in neutrophil surface CD11b expression, as well as a decrease in surface CD62L expression, was observed in patients with diabetic microangiopathy (37).

Chemotactic factors and their receptors also play a pivotal role in regulating the activation and movements of leukocytes through the extracellular matrix (28). In a polymicrobial infection, the CXCR2 blockade inhibits the neutrophil migration and increases the severity of sepsis (30). Moreover, in human (38) and experimental sepsis (14), the reduction in neutrophil chemotaxis is associated with decreased CXCR2 expression in neutrophils. In the current study, we found that blood neutrophils from diabetic mice submitted to MS had a significant decrease in CXCR2 expression in these cells compared with neutrophils from control mice. The levels of CXCR2 expression in diabetic mice were similar to those found in nondiabetic mice subjected to SS. Accordingly, neutrophils from diabetic mice subjected or not subjected to sepsis displayed decreased MIP-2–induced chemotaxis response. In vivo, MIP-2 administration in diabetic mice without sepsis also induced a low neutrophil migration.

Subsequently, we investigated whether the downregulation of CXCR2 expression in neutrophils during sepsis development in diabetic mice was mediated by GRK2 activation. GRK2 has been implicated in the downregulation of chemokine receptors (e.g., chemokine [C-C motif] receptors [CCRs] and CXCRs) and innate chemoattractant receptors (e.g., N-formylpeptide chemoattractant receptor [fMLP] and C5a receptor) (39,40). Moreover, increased levels of GRK2 in neutrophils from SS patients or mice are associated with decreased chemotactic responses (14,41). Notably, blood neutrophils from diabetic mice subjected to MS have significant increase in GRK2 expression compared with control mice.

However, despite the data presented here, a fundamental question remains unanswered. Which is the main factor that triggers the susceptibility of diabetic mice to sepsis? AGP is one of the major acute-phase proteins in humans and mice and has been described as an inhibitor of leukocyte functions, such as inhibition of rolling/adhesion and migration in vivo and the chemotaxis response in vitro (15). AGP serum concentration increases in response to systemic inflammation, infection, or diseases like diabetes (16,17,42). Interestingly, we found that AGP expression in serum of alloxan-treated diabetic mice started to increase at 2 h postsepsis and persisted at least until 12 h postsepsis (Fig. 4A and data not shown). Likewise, after MS induction in diabetic mice, serum levels of AGP were similar to those of nondiabetic mice subjected to SS. In addition, the hepatic AGP mRNA expression was significantly higher

in diabetic mice after CLP-induced sepsis. Moreover, when AGP was administrated to nondiabetic or diabetic mice treated with insulin, this protein inhibited the neutrophil migration to the focus of infection. These results suggest that AGP might play an important role as an immunosuppressing molecule associated with neutrophil migration failure and therefore in inducing the predisposition of diabetic mice to sepsis as well.

Other studies with diabetic rats and mice have also shown a decreased neutrophil migration (6,34,35), phagocytosis capacity (8), and hydrogen peroxide production (9). It should be emphasized that abnormalities in granulocyte chemotaxis, phagocytosis, and microbicidal activity have been described in poorly controlled, but not controlled, diabetic patients (31,43). Furthermore, the reduction of blood glucose levels by insulin treatment of diabetic patients (44) or rats (9) has been reported to be significantly correlated with improvement of neutrophil phagocytosis capacity. Here, we demonstrated that insulin treatment prevented the failure of neutrophil migration to the focus of infection, improved the infection control, and increased the survival rate of diabetic mice subject to sepsis by ~70% at 7 days after CLP. Moreover, insulin treatment inhibited AGP expression found in diabetic mice subjected to sepsis. As a consequence, the downregulation of CXCR2 and CD62L and the high expression of CD11b and GRK2 in neutrophils of diabetic mice subjected to sepsis were prevented. However, when AGP was administrated to diabetic mice treated with insulin, the protective effect of insulin was abolished.

The beneficial effect of insulin underpins that at least a moderate control of glucose levels by insulin administration enables diabetic mice to have a better profile of immune response. In contrast, treatment of control nondiabetic septic mice with insulin decreased survival rate. Insulin infusion has been used to normalize hyperglycemia; however, the beneficial effects of insulin and tight glucose control may be overcome by hypoglycemia and an increased death rate in critically ill patients (45). Therefore, further works are necessary to evaluate the adverse/beneficial effect of strict glucose control by insulin during sepsis.

In summary, our study provides evidence for a novel diabetes-insulin-sepsis-AGP axis in the regulation of neutrophil migration as well as control of susceptibility to infection. Moreover, these observations suggest that the regulation of AGP pathway, in conjunction with insulin therapy, merits further investigation as a potential immunopharmacologic intervention (monoclonal antibody) for enhancing the control of sensitivity to sepsis in diabetes.

REFERENCES

1. Smitherman KO, Peacock JE Jr. Infectious emergencies in patients with diabetes mellitus. Med Clin North Am 1995;79:53–77
2. Wu JH, Tsai CG. Infectivity of hepatic strain Klebsiella pneumoniae in diabetic mice. Exp Biol Med (Maywood) 2005;230:757–761
3. Ikejima S, Sasaki S, Sashinami H, et al. Impairment of host resistance to Listeria monocytogenes infection in liver of db/db and ob/ob mice. Diabetes 2005;54:182–189
4. Puliti M, Bistoni F, Orefici G, Tissi L. Exacerbation of group B streptococcal sepsis and arthritis in diabetic mice. Microbes Infect 2006;8:2376–2383
5. Martens GW, Arikan MC, Lee J, Ren F, Greiner D, Kornfeld H. Tuberculosis susceptibility of diabetic mice. Am J Respir Cell Mol Biol 2007;37:518–524
6. Sannomiya P, Oliveira MA, Fortes ZB. Aminoguanidine and the prevention of leukocyte dysfunction in diabetes mellitus: a direct vital microscopic study. Br J Pharmacol 1997;122:894–898
7. Delamaire M, Maugendre D, Moreno M, Le Goff MC, Allannic H, Genetet B. Impaired leucocyte functions in diabetic patients. Diabet Med 1997;14:29–34
8. Panneerselvam S, Govindasamy S. Sodium molybdate improves the phagocytic function in alloxan-induced diabetic rats. Chem Biol Interact 2003;145:159–163
9. Alba-Loureiro TC, Hirabara SM, Mendonça JR, Curi R, Pithon-Curi TC. Diabetes causes marked changes in function and metabolism of rat neutrophils. J Endocrinol 2006;188:295–303
10. Gupta S, Koirala J, Khardori R, Khardori N. Infections in diabetes mellitus and hyperglycemia. Infect Dis Clin North Am 2007;21:617–638, vii
11. Alves-Filho JC, de Freitas A, Spiller F, Souto FO, Cunha FQ. The role of neutrophils in severe sepsis. Shock 2008;30(Suppl 1):3–9
12. Tavares-Murta BM, Zaparoli M, Ferreira RB, et al. Failure of neutrophil chemotactic function in septic patients. Crit Care Med 2002;30:1056–1061
13. Alves-Filho JC, de Freitas A, Russo M, Cunha FQ. Toll-like receptor 4 signaling leads to neutrophil migration impairment in polymicrobial sepsis. Crit Care Med 2006;34:461–470
14. Alves-Filho JC, Freitas A, Souto FO, et al. Regulation of chemokine receptor by Toll-like receptor 2 is critical to neutrophil migration and resistance to polymicrobial sepsis. Proc Natl Acad Sci USA 2009;106:4018–4023
15. Mestriner FL, Spiller F, Laure HJ, et al. Acute-phase protein alpha-1-acid glycoprotein mediates neutrophil migration failure in sepsis by a nitric oxide-dependent mechanism. Proc Natl Acad Sci USA 2007;104:19595–19600
16. Poland DC, Schalkwijk CG, Stehouwer CD, Koeleman CA, van het Hof B, van Dijk W. Increased alpha3-fucosylation of alpha1-acid glycoprotein in Type I diabetic patients is related to vascular function. Glycoconj J 2001;18:261–268
17. Higai K, Azuma Y, Aoki Y, Matsumoto K. Altered glycosylation of alpha1-acid glycoprotein in patients with inflammation and diabetes mellitus. Clin Chim Acta 2003;329:117–125
18. Clark D. Guide for the Care and Use of Laboratory Animals. Washington, DC, National Academies Press, 1996, p. 21–70
19. Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock—a review of laboratory models and a proposal. J Surg Res 1980;29:189–201
20. Torres-Dueñas D, Celes MR, Freitas A, et al. Peroxynitrite mediates the failure of neutrophil migration in severe polymicrobial sepsis in mice. Br J Pharmacol 2007;152:341–352
21. Spiller F, Orrico MI, Nascimento DC, et al. Hydrogen sulfide improves neutrophil migration and survival in sepsis via K+ATP channel activation. Am J Respir Crit Care Med 2010;182:360–368
22. im Walde SS, Dohle C, Schott-Ohly P, Gleichmann H. Molecular target structures in alloxan-induced diabetes in mice. Life Sci 2002;71:1681–1694
23. Yu X, Park BH, Wang MY, Wang ZV, Unger RH. Making insulin-deficient type 1 diabetic rodents thrive without insulin. Proc Natl Acad Sci USA 2008;105:14070–14075
24. Gupta S, Chattopadhyay T, Pal Singh M, Surolia A. Supramolecular insulin assembly II for a sustained treatment of type 1 diabetes mellitus. Proc Natl Acad Sci USA 2010;107:13246–13251
25. Rajagopalan S. Serious infections in elderly patients with diabetes mellitus. Clin Infect Dis 2005;40:990–996
26. Saha JK, Xia J, Grondin JM, Engle SK, Jakubowski JA. Acute hyperglycemia induced by ketamine/xylazine anesthesia in rats: mechanisms and implications for preclinical models. Exp Biol Med (Maywood) 2005;230:777–784
27. Mastaitis JW, Wurmbach E, Cheng H, Sealfon SC, Mobbs CV. Acute induction of gene expression in brain and liver by insulin-induced hypoglycemia. Diabetes 2005;54:952–958
28. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 2007;7:678–689
29. Panés J, Perry M, Granger DN. Leukocyte-endothelial cell adhesion: avenues for therapeutic intervention. Br J Pharmacol 1999;126:537–550
30. Rios-Santos F, Alves-Filho JC, Souto FO, et al. Down-regulation of CXCR2 on neutrophils in severe sepsis is mediated by inducible nitric oxide synthase-derived nitric oxide. Am J Respir Crit Care Med 2007;175:490–497
31. Alves-Filho JC, Sônego F, Souto FO, et al. Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. Nat Med 2010;16:708–712
32. Miles AA, Miles EM, Burke J. The value and duration of defence reactions of the skin to the primary lodgement of bacteria. Br J Exp Pathol 1957;38:79–96
33. Mowat A, Baum J. Chemotaxis of polymorphonuclear leukocytes from patients with diabetes mellitus. N Engl J Med 1971;284:621–627
34. Pereira MA, Sannomiya P, Leme JG. Inhibition of leukocyte chemotaxis by factor in alloxan-induced diabetic rat plasma. Diabetes 1987;36:1307–1314
35. Fortes ZB, Farsky SP, Oliveira MA, Garcia-Leme J. Direct vital microscopic study of defective leukocyte-endothelial interaction in diabetes mellitus. Diabetes 1991;40:1267–1273
36. Petri B, Phillipson M, Kubes P. The physiology of leukocyte recruitment: an in vivo perspective. J Immunol 2008;180:6439–6446
37. Mastej K, Adamiec R. Neutrophil surface expression of CD11b and CD62L in diabetic microangiopathy. Acta Diabetol 2008;45:183–190
38. Chishti AD, Shenton BK, Kirby JA, Baudouin SV. Neutrophil chemotaxis and receptor expression in clinical septic shock. Intensive Care Med 2004;30:605–611
39. Langkabel P, Zwirner J, Oppermann M. Ligand-induced phosphorylation of anaphylatoxin receptors C3aR and C5aR is mediated by "G protein-coupled receptor kinases. Eur J Immunol 1999;29:3035–3046
40. Prossnitz ER, Kim CM, Benovic JL, Ye RD. Phosphorylation of the N-formyl peptide receptor carboxyl terminus by the G protein-coupled receptor kinase, GRK2. J Biol Chem 1995;270:1130–1137
41. Arraes SM, Freitas MS, da Silva SV, et al. Impaired neutrophil chemotaxis in sepsis associates with GRK expression and inhibition of actin assembly and tyrosine phosphorylation. Blood 2006;108:2906–2913
42. Baumann H, Gauldie J. The acute phase response. Immunol Today 1994;15:74–80
43. Alba-Loureiro TC, Munhoz CD, Martins JO, et al. Neutrophil function and metabolism in individuals with diabetes mellitus. Braz J Med Biol Res 2007;40:1037–1044
44. Jakelić J, Kokić S, Hozo I, Maras J, Fabijanić D. Nonspecific immunity in diabetes: hyperglycemia decreases phagocytic activity of leukocytes in diabetic patients. Med Arh 1995;49:9–12
45. Van den Berghe G, Schetz M, Vlasselaers D, et al. Clinical review: Intensive insulin therapy in critically ill patients: NICE-SUGAR or Leuven blood glucose target? J Clin Endocrinol Metab 2009;94:3163–3170