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Role of Acute-Phase Proteins in Interleukin-1-Induced Nonspecific Resistance to Bacterial Infections in Mice

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Treatment with a single low dose (80 to 800 ng) of interleukin-1 (IL-1) 24 h before a lethal bacterial challenge of granulocytopenic and normal mice enhances nonspecific resistance. Since IL-1 induces secretion of acute-phase proteins, liver proteins which possess several detoxifying effects, we investigated the role of these proteins in the IL-1-induced protection. Inhibition of liver protein synthesis with L-galactosamine (GALN) completely inhibited the IL-1-induced synthesis of acute-phase proteins. GALN pretreatment abolished the protective effect of IL-1 on survival completely (neutropenic mice infected with Pseudomonas aeruginosa) or partially (nonneutropenic mice infected with Klebsiella pneumoniae). Pretreatment with IL-6, a cytokine induced by IL-1, did not reproduce the protection offered after IL-1 pretreatment, nor did it enhance or deteriorate the IL-1-enhanced resistance to infection. A protective effect of IL-1 via effects on glucose homeostasis during the acute-phase response was investigated by comparing plasma glucose levels in IL-1-treated mice and control mice before and during infection. Although glucose levels in IL-1-pretreated mice were somewhat higher in the later stages of infection, no significant differences from levels in control mice were present, and the glucose levels in control-treated animals never fell to hypoglycemic values. We conclude that the IL-1-induced nonspecific resistance is mediated neither by the induction of IL-6 nor by the effects of IL-1 on glucose homeostasis. Acute-phase proteins generated after IL-1 pretreatment, however, seem to play a critical role in the IL-1-induced protection to infection.

Administration of the proinflammatory cytokine interleukin-1 (IL-1) has been shown to enhance nonspecific resistance in animals to several gram-positive and gram-negative bacteria, fungi, and plasmodia (35). The exact mechanism of this protection is still unclear. A direct antimicrobial effect of IL-1 in vitro (30) has been excluded, and enhanced clearance of microorganisms induced by IL-1 in vivo has not been a constant finding (22, 30). The protective effect of IL-1 to infectious challenges in granulocytopenic mice indicates that an effect on neutrophils does not mediate the IL-1-induced resistance (30, 35). Additional studies have provided evidence against a major role of IL-1-induced cyclooxygenase metabolites, glucocorticosteroids, or cytokines like IL-6 or tumor necrosis factor (TNF) in the IL-1-induced protection (30, 33–35).

In the present study, we investigated whether the effects of IL-1 on the acute-phase response could be responsible for the increased resistance induced by IL-1. The acute-phase response comprises a constellation of metabolic, endocrinologic, neurologic, and immunologic alterations which starts within hours of an initiating stimulus. These changes appear to be meant to balance or curtail the potential adverse consequences of the inflammatory process and restore homeostasis (15). Synthesis of acute-phase proteins by the liver is a major component of the acute-phase response, and IL-1 appears to be an important inducer. We addressed the issue of whether enhanced resistance to infection after preexposure to IL-1 might be due to IL-1-induced enhanced secretion of acute-phase proteins using L-galactosamine (GALN), a liver-specific blocker of RNA and protein synthesis which acts by depleting UTP and thereby is able to block protein synthesis in the acute phase of infection (5). We investigated the extent to which GALN inhibits the IL-1-induced acute-phase protein secretion by the liver. Next, we assessed whether GALN would also inhibit the IL-1-induced enhanced survival of normal and neutropenic mice after a lethal gram-negative bacterial challenge. Since IL-1 has been shown to induce IL-6 production by endothelial cells, fibroblasts, macrophages, and other cell types, it has been proposed that IL-6 is the direct mediator of a number of activities that were originally attributed to IL-1 (11, 32). IL-6 induces a series of acute-phase proteins and in addition possesses some other potentially protective effects during an infection (12, 32). Hence, we studied the role of IL-6 in IL-1-induced protection.

Another component of the acute-phase response to trauma and infection is carbohydrate dyshomeostasis. During severe infection, especially in rodents, an initial hyperglycemia may devolve into a profound hypoglycemia, the latter being a sign of decompensation (7). Since IL-1 affects glucose metabolism in various ways (29), we addressed the question of whether the effects of IL-1 on glucose homeostasis resulted in enhanced survival.

(Materials and Methods: Mice. Female outbred Swiss mice (weight, 20 to 25 g; TNO, Rijswijk, The Netherlands) were kept under pathogen-free conditions with a light-dark cycle of 12 h (lights on at 7 a.m., lights off at 7 p.m.). Standard lab chow (RMH-TM; Hope Farms, Woerden, The Netherlands) and

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acidiﬁed water were available ad libitum. Granulocytopenic mice received irradiated food.

Materials. Recombinant human IL-1ß (rhIL-1ß) was kindly provided by P. Graber (Glaxo, Geneva, Switzerland) and P. Ghiara (Sciavo, Siena, Italy). According to the specifications of the suppliers, endotoxin contamination was negligible (<1.53 and <1.2 ng/mg, respectively). Recombinant human IL-6 (rhIL-6) was generously supplied by P. Stutz, Sandoz Research Institute, Vienna, Austria. Endotoxin contamination was <0.4 U/mg in the Limulus assay. Recombinant murine IL-6 (rmIL-6) was a kind gift from J. L. Renauld, Ludwig Institute for Cancer Research, Brussels, Belgium. Cytokines were stored undiluted at −70°C (rhIL-1ß and rhIL-6) or 4°C (rmIL-6) and were diluted immediately before use in pyrogen-free isotonic phosphate-buffered saline (PBS; pH 7.4). Inactivated IL-1 was prepared by heating IL-1 at 95°C for 30 min. To all these solutions, normal mouse serum was added to an end volume of 2% (vol/vol). GALN was purchased from Merck, Darmstadt, Germany, and was dissolved in pyrogen-free saline shortly before injection. Endotoxin content was <38 ng of GALN per g. Gentamicin was purchased from Schering, Kenilworth, N.J. Cyclophosphamide (ASTA Pharma, Frankfurt, Germany) was dissolved in sterile pyrogen-free distilled water. Bacteria for infectious challenges (Klebsiella pneumoniae ATCC 43816 and Pseudomonas aeruginosa ATCC 27853) were cultured overnight and washed three times in saline, and appropriate dilutions were prepared.

Experimental procedures. All experiments started at 9:30 a.m. (time zero).

(i) Liver enzymes, acute-phase proteins, and IL-6 and glucose levels after treatment with IL-1 and/or GALN. For assessment of liver injury by GALN, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) levels were determined in sera obtained from mice 24 h after intraperitoneal (i.p.) injection of different doses of GALN, 400 ng of rhIL-1ß, or PBS. For determinations of acute-phase proteins and IL-6 levels, mice received two injections. GALN or vehicle (saline) was given i.p. at −30 min, and IL-1ß or control (heat-inactivated IL-1) was administered i.p. (0.1 ml) at time zero. At various time points, blood was taken retro-orbitally from ether-anesthetized mice to prepare EDTA-treated plasma (fibrinogen measurements) or serum (serum amyloid A [SAA] and IL-6 measurements). IL-6 levels were also measured in serum obtained at different time points after a single i.p. treatment with 800 ng of rhIL-1ß or 800 ng of rhIL-6.

In the experiments in which glucose was measured, mice received a single i.p. injection of 800 ng of IL-1 or vehicle at time zero, an intramuscular (i.m.) challenge with K. pneumoniae at 24 h, and gentamicin (120 mg/kg subcutaneously) 6 h later. Trunk blood of decapitated mice was collected at several time points in fluoride-coated tubes on melting ice. All plasma and serum samples were stored at −20°C until assay.

When neutropenic mice were used, neutropenia (<0.5 × 10⁹ granulocytes per liter) was induced by two subcutaneous injections of cyclophosphamide (150 and 100 mg/kg, respectively) on days −3 and 0.

(ii) Effects of cytokine and/or GALN pretreatment on survival. For survival experiments, some mice were rendered neutropenic as described above. IL-1 or control treatment (heat-inactivated IL-1) was administered i.p. (0.1 ml) at time zero. GALN (18 mg per mouse) or vehicle (saline) was given i.p. 30 min earlier. The infectious challenge was administered at 24 h. Neutropenic mice received P. aeruginosa i.m. (0.5 × 10⁸ to 1 × 10⁸ CFU per mouse). Because P. aeruginosa does not kill nonneutropenic mice unless very high inocula are used, such mice received K. pneumoniae (1 × 10⁶ to 5 × 10⁵ CFU per mouse). Six hours after the infectious challenge, 120 mg of gentamicin per kg of body weight was given subcutaneously in order to postpone the time of death and thus accentuate the differences between treatment groups. Mortality was recorded for a period of at least 48 h after challenge.

In investigations on the protective effect of human and murine IL-6 on P. aeruginosa infection in neutropenic mice, the experimental protocol described above was used. The cytokines were given i.p. (0.1 ml) at time zero, and when both IL-1 and IL-6 were administered, a mixture of both cytokines was prepared immediately before injection. In all cases, heat-inactivated IL-1ß was given as the control treatment.

Assays. Serum ASAT and ALAT levels were assessed by routine procedures (BM/Hitachi 747; Boehringer Mannheim, Germany). Plasma glucose concentrations were determined by the hexokinase method (Cobas Bio; Hoffmann-La Roche B.V., Mijdrecht, The Netherlands). IL-6 levels in serum were quantitated as hybridoma growth factor activity by using the IL-6-dependent murine hybridoma 7TD1 (a kind gift from J. van Snick) as described previously (28). Hybridoma growth factor activity resulting in half-maximal stimulation of the target cell growth was arbitrarily defined as 1 U. The reference standard in these experiments was rhIL-6 (specific activity, 10⁷ U/ml; a kind gift from Immunex Corp., Seattle, Wash.). The detection limit of the assay was 50 U/ml. SAA was measured by a modification of the direct-binding enzyme-linked immunosorbent assay method for human plasma (27) by using a mixture of rabbit polyclonal antiserum to mouse SAA, and SAA1 (kind gift from J. Sipe, Boston, Mass.). Assays for fibrinogen in EDTA-plasma were performed by measuring the rate of conversion of fibrinogen to fibrin in the presence of thrombin excess. Measurements of fibrin clot formation were performed on a coagulometer (KC-10; Salm & Kipp, Breukelen, The Netherlands). The results are expressed as milligrams of fibrinogen per liter of plasma; the sensitivity of the assay was 1,200 mg/liter. Endotoxin levels were assessed by the Limulus amebocyte lysate gelation test, which had a sensitivity of 6 pg/ml.

Statistical analysis. Survival curves were analyzed by the log rank test (24). Comparisons of medians of liver enzymes, acute-phase proteins, IL-6 levels, and glucose levels between treatment groups were made by the Kruskal-Wallis test at each time point (13). When five components were analyzed, the Bonferroni-corrected α = 0.05/5 = 0.01 was used.

RESULTS

Hepatotoxicity of GALN. Since GALN is potentially hepatotoxic (5), we first assessed the effect in Swiss mice of different doses of this hexosamine on ASAT and ALAT levels in serum at 24 h after i.p. injection. At this time point, liver damage should have developed (5). After administration of 18 and 36 mg of GALN per mouse (720 and 1,440 mg/kg, respectively), doses which are commonly used by others (1, 18, 37) and which are reported to be effective in the inhibition of protein synthesis (1, 5), serum ASAT and ALAT levels in normal mice did not differ significantly from those in vehicle-treated mice; 90 and 180 mg of GALN per mouse induced a significant increase in the levels of both enzymes compared with the levels in vehicle-treated mice.
(Fig. 1). In neutropenic mice, ASAT and ALAT levels in mice after administration of 18 mg of GALN did not differ significantly from those in vehicle-treated mice; ASAT levels were elevated significantly compared with those in vehicle-treated mice after administration of 180 mg of GALN ($P < 0.0005$), and ALAT levels were significantly increased after administration of 36 and 180 mg of GALN ($P < 0.01$ and $P < 0.001$, respectively) (data not shown). Also, ASAT and ALAT levels in normal and neutropenic mice 24 h after treatment with two i.p. injections of 18 mg of GALN or vehicle and treatment with 800 ng of IL-1 or control treatment (the experimental setting to be used) were not significantly increased (data not shown). In further experiments, a dose of GALN of 18 mg per mouse was used.

**Inhibition of IL-1-induced increase of acute-phase protein production by pretreatment with GALN.** To verify the effect of GALN as an inhibitor of acute-phase protein synthesis, concentrations of SAA in serum and concentrations of fibrinogen in plasma were chosen as markers of the acute-phase response after IL-1 treatment. These acute-phase proteins were measured in normal and neutropenic mice at the times that they were at their peak levels, at 8 and 24 h, respectively, after IL-1 administration (3, 9), with or without i.p. pretreatment with GALN 30 min before IL-1 treatment. As shown in Fig. 2 for nonneutropenic mice, 800 ng of IL-1 administered i.p. induced SAA at 8 h and fibrinogen at 24 h. The induction by IL-1 of SAA in normal (Fig. 2A) and neutropenic mice (peak level, 162 μg/liter; six mice per group) was completely inhibited by GALN pretreatment ($P < 0.001$), with no significant difference between SAA levels in mice treated with GALN plus the control treatment and mice treated with GALN plus IL-1.

Plasma fibrinogen levels in GALN-treated normal mice were lower than those in vehicle-treated mice ($P < 0.01$; Fig. 2B). The significant increase in plasma fibrinogen levels induced by treatment with 800 ng of IL-1 ($P < 0.005$) was completely inhibited by pretreatment with GALN ($P < 0.01$), and fibrinogen concentrations in mice treated with GALN plus IL-1 did not differ significantly from those in mice treated with GALN plus inactivated IL-1.

Results similar to those described above were observed in neutropenic mice. Pretreatment of these mice with GALN before the IL-1 injection significantly inhibited the plasma fibrinogen level increase in comparison with levels in mice that received vehicle before the IL-1 injection (median fibrinogen levels, 3,000 mg/liter after IL-1 treatment and 1,425 mg/liter after treatment with both GALN and IL-1; $P < 0.001$). Median fibrinogen levels in mice after GALN plus IL-1 treatment did not differ significantly from those in mice treated with GALN plus inactivated IL-1. Fibrinogen concentrations in GALN-treated mice were somewhat lower than those in vehicle-treated mice (1,245 and 1,725 mg/liter, respectively), but the difference was not significant.

**Effects of pretreatment with IL-1 and/or GALN on survival.** Pretreatment of nonneutropenic mice with 800 ng of IL-1 24 h before an i.m. infection with *K. pneumoniae* significantly enhanced survival ($\chi^2 = 25.93$; $P < 0.0005$) (Fig. 3A). Administration of GALN 30 min before the IL-1 pretreat-
ment partially inhibited the IL-1-induced protection, with significant differences in this group compared with the levels of protection in both the IL-1-treated group and the group treated with GALN alone. Serum IL-6 levels were measured. IL-6 levels peaked at 30 min after an i.p. injection of 800 ng of rhIL-6 (38,900 U/ml) and at 2 h after i.p. injection of 800 ng of IL-1 (5,020 U/ml) (six mice per group). However, no protective effect of pretreatment with 80, 400, or 800 ng of IL-6 was observed (Fig. 4A). To investigate whether some degree of species specificity of IL-6 (17, 21) plays a role, we also assessed the effect of mIL-6 (80, 400, 800, and 1600 ng) on survival of P. aeruginosa infection in neutropenic mice, but this molecule also did not influence survival (Fig. 4B).

Since several studies report synergistic effects between IL-1 and IL-6 (4, 11), we assessed whether IL-6 would enhance IL-1-induced protection to a lethal P. aeruginosa infection. However, administration of 80 or 800 ng of hIL-6 or mIL-6 did not affect protection induced by 80 or 800 ng of IL-1 (shown for mIL-6 in Fig. 4C).

The deleterious effects of IL-6 in infections have also been suggested in several reports (31, 36). We addressed this issue by administering higher doses of IL-6 prior to P. aeruginosa infection, but we observed that survival of mice pretreated with rhIL-6 doses of 1.6, 4, and 8 μg and 1.6 μg of rhIL-6 per mouse did not differ significantly from survival of control-treated mice (Fig. 4D). Because of the complete absence of an effect of IL-6, we did not perform similar studies in nonneutropenic mice.

**Effect of IL-1 treatment on plasma glucose concentration before and after infection.** Experiments examining the effect of IL-1 treatment on the plasma glucose concentration before and after infection were limited to mice infected with K. pneumoniae, because fewer pretreatments, which could possibly influence glucose metabolism, are necessary in this model. The plasma glucose concentrations (median values) in mice after an i.p. injection of 800 ng of IL-1 at time −24 h and K. pneumoniae infection by i.m. injection of K. pneumoniae at time zero are depicted in Fig. 5. The plasma glucose concentration showed a nadir at 4 h after i.p. injection in IL-1-treated mice but not in vehicle-treated mice (4.85 and 8.5 mmol/liter, respectively). The difference between IL-1-treated and vehicle-treated mice was significant at 4 and 12 h after IL-1 injection; and also at 24 h after IL-1 injection, the time point of infectious challenge, the glucose level was significantly lower than that in control mice. The glucose levels in IL-1-treated mice returned to control levels at 36 h after IL-1 injection.

Median plasma glucose levels after i.m. infection with K. pneumoniae did not differ significantly between the treatment groups, with the exception of the level at 2 h, when values in the IL-1-treated group were still lower, and with the level at 48 h after infection, when values in the IL-1-treated group were higher. In late stages of the infection, there is a tendency for mice pretreated with IL-1 to have higher glucose levels than those in mice given the control treatment. At this stage, signs of discomfort in the infected animals (piloerection, decreased physical activity) were also less pronounced in the IL-1-treated group. The IL-1-pretreated mice all survived for 48 h, whereas approximately 20% of the control-treated mice died during this period.

**DISCUSSION**

We have previously reported that a single low dose of IL-1 administered 24 h, but not 6 or 0.5 h, before a lethal
gram-negative bacterial infection enhances survival (30, 32a). In the present study, we demonstrated that this enhanced survival is associated with an increased level of production of acute-phase proteins. Acute-phase proteins, which are synthesized by the liver during a variety of acute inflammatory processes (15), may operate as oxygen radical scavengers, protease inhibitors, coagulation factors, and opsonins, and may therefore play a role in the nonspecific defense of the host to infections. Most acute-phase proteins are induced by IL-1 within a time course similar to that required for induction of protection in a lethal infection. To investigate the role of acute-phase proteins in the IL-1-induced enhanced resistance, we used GALN, a hepatocyte-specific inhibitor of RNA and protein synthesis (5). In normal and neutropenic mice, the IL-1-induced synthesis of acute-phase proteins was completely blocked by GALN pretreatment. The protective effect of IL-1 pretreatment on infection was inhibited partially (nonneutropenic mice infected with K. pneumoniae) or completely (neutropenic mice infected with P. aeruginosa) by a GALN dose that did not damage liver parenchymal cells. GALN did not inhibit production of IL-1-induced IL-6, confirming the expected specificity of the effects of GALN on hepatocytes (which do not produce IL-6), not affecting protein synthesis in IL-6-producing cells. IL-6 concentrations in mice treated with GALN and IL-1 were even somewhat enhanced in comparison with those in mice treated with only IL-1 (2.7-fold increase in normal mice; 1.2-fold increase in neutropenic mice). GALN per se did not increase IL-6 levels, arguing against an effect of traces of contaminating endotoxin (less than 0.7 pg per mouse). GALN itself may have a slight priming effect on macrophage cytokine production, as observed by some investigators in vitro (16) and in vivo (18) after an endotoxin trigger. Others did not find such an effect (1). We do not believe that a GALN-induced sensitizing effect of macrophages for IL-1, resulting in increased mac-
crease in the level of IL-1-induced protection to infection, since the IL-6/GALN inhibited enhanced survival of a lethal dose of toxin and TNF toxicities was shown to be mediated by inhibition of hepatic metabolism and not by activation of macrophages, since this sensitization can be completely reversed by restoring hepatic RNA and protein syntheses with uridine supplementation (1, 8, 19).

The lack of an effect of GALN pretreatment on survival of infection per se also argues against a detrimental effect of GALN via mechanisms independent of IL-1-induced protection, e.g., by an aspecific toxic effect of GALN or by GALN-induced sensitization for bacterial products or cytokines like TNF (8, 37), and against a deleterious effect of the reduced serum fibrinogen levels observed in GALN-treated mice. Taken together, these results suggest a critical role of liver-induced proteins, most conceivably acute-phase proteins, in the IL-1-induced nonspecific resistance to infection. A role of acute-phase proteins in IL-1-induced enhanced survival of several challenges has also been suggested by other investigators. GALN abolished IL-1-induced protection to a subsequent lethal dose of IL-1 or TNF (37), and GALN inhibited enhanced survival of a lethal dose of lipopolysaccharide or TNF after a turbentine-induced acute-phase response in mice (1). Libert and coworkers (19) showed an inhibition by GALN of the protective effect of IL-1 pretreatment to a lethal challenge of TNF-α and GALN, an inhibition which could be restored by the addition of uridine. Those authors also suggested a role of acute-phase proteins in the IL-1-induced protection (19).

IL-1 induces IL-6, and several effects of IL-1 have been reported to be mediated by IL-6 (11, 32). Apart from induction of acute-phase proteins, IL-6 has other potentially beneficial effects (6, 14, 26, 32). Nevertheless, our observations plead against a role of IL-6 in the IL-1-induced enhanced resistance to infection. First, although GALN inhibits IL-1-induced protection, it does not inhibit IL-1-induced IL-6 production. Second, doses of 80 to 800 ng of human IL-6 did not enhance survival after a lethal P. aegyptius challenge, despite the larger IL-6 peak in mice after rhIL-6 treatment than that after IL-1 treatment. Higher doses (1.6 to 8 µg) neither protected nor showed deleterious effects, in accordance with earlier reports by us and others (4, 32). Since there is some species specificity for IL-6 (17, 21), we tested the protective effect of pretreatment with different doses of mIL-6, but no effect on survival was observed. Some studies describe a synergetic effect between IL-1 and IL-6 (4, 11), and IL-1 and IL-6 are likely to be present simultaneously after IL-1 injection. However, we found that coadministration of different doses of rhIL-6 or mIL-6 did not enhance the IL-1-induced enhanced survival of infection.

Other investigators reported protection by IL-6 pretreatment against a lethal combination of lipopolysaccharide and GALN, provided that the induced TNF was partially neutralized (2). Using very high doses of mIL-6 (20 to 50 µg), another group found enhanced clearance of Listeria monocytogenes (20). However, in accordance with our results, pretreatment with 0.2 to 5 µg of mIL-6 did not protect against a lethal combination of recombinant murine TNF and GALN, whereas IL-1 did, according to the authors (19), by induction of acute-phase proteins. In addition, our results showing high IL-6 levels associated with the lack of protection to infection in mice pretreated with GALN and IL-1 are similar to the high serum IL-6 levels reported by Havell and Sehgal (10) in anti-TNF-α-treated mice that succumbed to experimental listeriosis. The fact that IL-6 as an inducer of acute-phase proteins is not capable of enhancing nonspecific resistance, whereas acute-phase proteins themselves seem to do so, is not necessarily contradictory. IL-6 and IL-1 are known to induce different spectra of acute-phase proteins (25), and it is conceivable that the IL-1-induced spectrum is protective and the IL-6-induced spectrum is not. Furthermore, IL-1 is a more potent inducer than IL-6 for several proteins that can be induced by both IL-1 and IL-6 (21, 23, 25).

We also investigated the role of IL-1-induced changes in blood glucose levels. IL-1 affects glucose homeostasis in different ways (7, 29). Whereas insulin-inducing and insulin-like effects would lead to hypoglycemia, the toxic effects of higher doses of IL-1 on pancreatic β cells and the induction by IL-1 of glucagon and stress hormones would elevate plasma glucose levels. These effects may result in a reduction of hypoglycemia during the terminal phase of sepsis. Although the fall in blood glucose levels induced by IL-1 was not completely restored at the time of infection in our mice, the plasma glucose levels measured in infected mice after IL-1 treatment did not differ significantly from those in controls at most time points. Moreover, in both treatment groups, glucose levels never fell to hypoglycemic values. Therefore, effects on blood glucose levels do not seem to play a major role.

In conclusion, the data presented here suggest that the increase in resistance induced by IL-1 is at least partially mediated by GALN-inhibitable liver proteins, most likely acute-phase proteins. IL-6 does not seem to be an intermediate for this IL-1 effect. Finally, the improved outcome of infection in IL-1-treated mice is not associated with physiologically significant changes in blood glucose levels during infection.

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