Leukotriene B₄–mediated sterile inflammation promotes susceptibility to sepsis in a mouse model of type 1 diabetes

Luciano Ribeiro Filgueiras,¹,² Stephanie L. Brandt,¹ Soujuan Wang,¹ Zhuo Wang,¹ David L. Morris,³ Carmella Evans-Molina,³ Raghavendra G. Mirmira,³ Sonia Jancar,² C. Henrique Serezan¹*

Type 1 diabetes mellitus (T1DM) is associated with chronic systemic inflammation and enhanced susceptibility to systemic bacterial infection (sepsis). We hypothesized that low insulin concentrations in T1DM trigger the enzyme 5-lipoxygenase (5-LO) to produce the lipid mediator leukotriene B₄ (LTB₄), which triggers systemic inflammation that may increase susceptibility to polymicrobial sepsis. Consistent with chronic inflammation, peritoneal macrophages from two mouse models of T1DM had greater abundance of the adaptor MyD88 (myeloid differentiation factor 88) and its direct transcriptional effector STAT-1 (signal transducer and activator of transcription 1) than macrophages from nondiabetic mice. Expression of Alox5, which encodes 5-LO, and the concentration of the proinflammatory cytokine interleukin-1β (IL-1β) were also increased in peritoneal macrophages and serum from T1DM mice. Insulin treatment reduced LTB₄ concentrations in the circulation and Myd88 and Stat1 expression in the macrophages from T1DM mice. T1DM mice treated with a 5-LO inhibitor had reduced Myd88 mRNA in macrophages and increased abundance of IL-1 receptor antagonist and reduced production of IL-β in the circulation. T1DM mice lacking 5-LO or the receptor for LTB₄ also produced less proinflammatory cytokines. Compared to wild-type or untreated diabetic mice, T1DM mice lacking the receptor for LTB₄ or treated with a 5-LO inhibitor survived polymicrobial sepsis, had reduced production of proinflammatory cytokines, and had decreased bacterial counts. These results uncover a role for LTB₄ in promoting sterile inflammation in diabetes and the enhanced susceptibility to sepsis in T1DM.

INTRODUCTION

Imbalances between metabolism and the immune system have been linked with inflammatory diseases such as atherosclerosis, obesity, gout, and diabetes (1). Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by destruction of pancreatic β cells, leading to deficiency in the production of insulin and consequent hyperglycemia (2). Either hyperglycemia or lack of insulin could lead to a chronic proinflammatory state characterized by increased concentrations of inflammatory cytokines in the serum such as interleukin-2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-1β (3, 4). Enhanced production of proinflammatory cytokines in the absence of an infectious agent is called sterile inflammation (5, 6). Sterile inflammation contributes to different metabolic diseases such as T1DM, gout, and atherosclerosis (7–9). It is likely that T1DM-induced alterations in the innate and adaptive components of the immune system play a critical role in the accelerated development of cardiovascular disease and other complications of diabetes, such as enhanced susceptibility to local and systemic (sepsis) infection, a condition frequently found in persons with diabetes (10–12).

Sepsis is a multifactorial disease initially involving a systemic uncontrolled production of inflammatory mediators (also known as a “cytokine storm”), termed systemic inflammatory response syndrome (SIRS), in response to microbial infection (13, 14). In view of the enhanced inflammatory state found in diabetes, it seems possible that a predisposition to development of SIRS may produce septic shock and death in septic diabetic individuals. However, whether susceptibility to SIRS is the predominant driver involved in enhanced susceptibility to sepsis in T1DM remains to be determined.

Among the mediators produced by activated macrophages, the secretion of the bioactive lipid leukotriene B₄ (LTB₄), which is generated by 5-lipoxygenase (5-LO), enhances the antimicrobial effector functions and cytokine production of macrophages (15, 16). LTB₄ has been previously characterized as a phagocyte chemoattractant that elicits both acute inflammatory responses and maintenance of chronic inflammation (15, 16). We and others have shown that LTB₄ signaling through its cognate receptor B leukotriene receptor 1 (BLT1) leads to a Gαi-mediated decrease in cyclic adenosine monophosphate (cAMP) concentrations and enhances macrophage effector function (16–20). Inhibition of cAMP production increases pathogen recognition receptor (PRR) activation of the proinflammatory transcription factor nuclear factor κB (NFκB) (21, 22) and consequent production of cytokines including TNF-α (22, 23). Among PRRs, Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns such as lipopolysaccharides (LPSs) from Gram-negative bacteria. TLR family members and the IL-1β receptor (IL-1R) share a conserved cytoplasmic Toll–IL-1R (TIR) domain that binds adaptor proteins, including myeloid differentiation factor 88 (Myd88) (24). Myd88-mediated signaling through all known TLRs except TLR3, but its importance for individual TLRs varies (24). Although it is crucial for initiating signaling responses to IL-1β and other members of the interleukin family, such as IL-18, Myd88 does not mediate responses to other cytokines, such as TNF-α (24). Myd88 expression is enhanced by proinflammatory substances, such...
RESULTS

Macrophage STAT-1 and MyD88 abundance are enhanced in mice models of T1DM

Because T1DM is accompanied by a constitutive low-grade inflammatory response, we speculated that T1DM mice would exhibit high MyD88 abundance, allowing the inflammatory response (4, 28–30). Initially, we determined the expression of Myd88 and Stat1 in macrophages from streptozotocin (STZ)–treated mice. This model resembles many aspects of the T1DM, such as low insulin production and hyperglycemia (31, 32). Ten days after the induction of diabetes, mice had similar body weights but higher glucose concentration and lower insulin concentrations than control mice (fig. S1, A to D). Myd88 and Stat1 mRNA and protein abundance were higher in resident peritoneal macrophages from STZ-treated mice and mice with genetically induced T1DM (nonobese diabetic [NOD]/ShiLtJ mice) than those from control mice (Fig. 1, A to C). Similarly, Myd88 and Stat1 expression was higher in alveolar macrophages from STZ–treated mice (fig. S2). The expression of mRNAs encoding other TIR adaptors such as TIR-containing adapter molecule (Ticam) and TIR domain–containing adapter–inducing IFN-β (Trif) did not differ in macrophages from diabetic NOD mice or control ICR/HAL mice (Fig. 1D), suggesting that the abundance of Myd88 and Stat1 in macrophages is specifically altered in two different T1DM murine models. We then determined whether macrophages from diabetic mice had increased responsiveness to the IL-1R agonist IL-1β and TLR4 agonist LPS, the responses to which require MyD88 to elicit macrophage activation (24, 33). Macrophages were deemed to be more responsive to TLRs as LTB4 (25–27). We and others have shown that Stat1 (signal transducer and activator of transcription 1) is the main transcription factor involved in Myd88 expression in macrophages (21).

Thus, we aimed to elucidate the molecular mechanisms involved in sterile inflammation and increased sepsis susceptibility in T1DM. We hypothesized that the low-grade inflammation that occurs in T1DM is promoted by LTB4, which enhances IL-1R and TLR responsiveness. We found that Myd88 and Stat1 expression was enhanced in mice models of T1DM through constitutive LTB4 production. Additionally, we found that LTB4 increased IL-1β production and decreased IL-1RA abundance, both of which favor IL-1R activation. Collectively, our findings show that enhanced LTB4 production increases proinflammatory cytokine production and responsiveness to MyD88-dependent receptors. Moreover, our results show that the LTB4–BLT1 axis is involved in enhanced susceptibility to polymicrobial sepsis in diabetic mice.

Fig. 1. Expression and responsiveness to Myd88 and STAT-1 are enhanced in macrophages from type 1 diabetic mice. (A) Myd88 and Stat1 mRNA expression in resident peritoneal macrophages from control and STZ diabetic mice was determined by real-time reverse transcription polymerase chain reaction (RT-PCR). (B) STAT-1, Myd88, and β-actin protein abundance in resident macrophages from control and STZ diabetic mice was determined by Western blot. Relative Myd88 and STAT-1 abundance was determined by densitometry (left); immunoblot results are representative of three independent experiments. Values for the wild-type (WT) control group were set as 100%. (C) Myd88 and Stat1 mRNA expression in resident macrophages from NOD/ShiLtJ and control nondiabetic ICR/HAL mice was determined by real-time PCR. (D) Ticam and Trif mRNA expression in resident peritoneal macrophages from NOD/ShiLtJ and control nondiabetic ICR/HAL mice was determined by real-time RT-PCR. (E to J) Resident peritoneal macrophages from STZ–treated and control (vehicle) mice were challenged with either IL-1β (E and F) or LPS (G to I), and cells or supernatants were harvested for determination of mRNA expression for Myd88 (E and G), Stat1 (H), Nos2 (I), or nitrite concentrations (F and J). In all circumstances, data are presented as means ± SEM from at least three independent experiments with at least three mice per experimental group. *P < 0.05 compared to vehicle, LPS, or IL-1β; †P < 0.05 compared to vehicle-treated mice or ICR/HAL mice or vehicle control; ‡P < 0.05 compared to vehicle, LPS, or IL-1β; ‡P < 0.05 compared to nonstimulated macrophages from STZ–treated mice.
and IL-1R if Myd88 and Stat1 expression was increased, and if nitric oxide (NO) production was increased, because the gene encoding inducible NO synthase (Nos2) is a target of NFκB (34). NO is produced by activated macrophages and is an essential microbicidal molecule (35–39). IL-1β exposure increased Myd88 expression and NO production in macrophages from T1DM mice (Fig. 1, E and F). Similarly, LPS exposure increased Stat1 and Myd88 expression (Fig. 1, G and H). We detected increased expression of Nos2 mRNA and increased NO production in macrophages from diabetic mice under basal conditions, indicating that STZ-induced diabetes skews macrophages toward a heightened inflammatory phenotype (Fig. 1, I and J). These data show that in two independent murine models of T1DM, macrophages exhibited high basal and inducible Myd88 and Stat1 expression, leading to enhanced TLR4 and IL-1R1 responsiveness.

**LTB₄-BLT1 mediates enhanced Myd88 expression in macrophages from type 1 diabetic mice**

We have previously shown that LTB₄ enhances Stat1-dependent Myd88 expression in macrophages (21). On the basis of this result, we speculated that enhanced Myd88 and Stat1 expression in T1DM might be mediated by constitutive LTB₄ production. LTB₄ concentrations were higher in both macrophages and serum of STZ-treated or diabetic NOD mice compared to those in nondiabetic control mice (Fig. 2, A and B). We next determined the expression of the mRNAs encoding the LT-generating enzyme (Alox5) and the LTB₄ receptor (Ltb4r1). Alox5 expression was increased in macrophages from STZ-treated mice compared to controls, whereas Ltb4r1 expression was similar in both STZ-treated and control mice (Fig. 2C). To determine the roles of LTB₄ and BLT1 in controlling Myd88 and Stat1 expression in T1DM, we induced T1DM in 5-LO⁻/⁻ or BLT1⁻/⁻ mice. As expected, 5-LO⁻/⁻ and BLT1⁻/⁻ macrophages from untreated mice exhibited lower Myd88 and Stat1 expression (Fig. 2, D and E). Myd88 and Stat1 expression was not enhanced in macrophages from 5-LO⁻/⁻ or BLT1⁻/⁻ T1DM mice (Fig. 2, D and E). Furthermore, insulin or blood glucose concentrations did not differ between STZ-treated 5-LO⁻/⁻ mice, BLT1⁻/⁻ mice, or diabetic mice treated with 5-LO inhibitor (Fig. S1, B, C, and F), indicating that 5-LO deficiency and BLT1 actions in Myd88 and Stat1 expression are not due to changes in hyperglycemia or insulin in T1DM. Next, we determined whether the activity of the transcription factor cJun, which can activate Stat1 expression (40), was stimulated by LTB₄ and whether cJun promoted Stat1 transcription. Phosphorylation of Ser³²⁵ in cJun (a phosphorylation event that is essential for its transcriptional activity) (41), but not that of Ser³⁸³, was enhanced in macrophages from diabetic wild-type mice, but not in macrophages from 5-LO⁻/⁻ diabetic mice, suggesting that LTs promote cJun transcriptional activity (Fig. 2F). Moreover, in macrophages from diabetic mice, cJun bound two different regions of the Stat1 promoter, an event that was blunted in diabetic 5-LO⁻/⁻ mice (Fig. 2G). We have previously shown that silencing of Stat1 abrogates LT₄-induced Myd88 expression in macrophages (21). Here, we sought to determine whether LTB₄ promoted Stat1 binding to the Myd88 promoter. Treatment of macrophages with LTB₄ increased binding of Stat1 to Myd88 promoter about threefold, as shown by chromatin immunoprecipitation (ChIP) assay (Fig. 2H). We also tested two other promoter regions, but LT₄-mediated Stat1 binding to the Myd88 promoter was specific to a single region (Fig. 2H). Together, these findings show that basal LTB₄ production in T1DM increases Myd88 expression in a manner dependent on cJun-mediated Stat1 expression.

**LTB₄ controls systemic inflammation in T1DM**

Because most morbidities associated with type 1 and type 2 diabetes are due to the basal low-grade inflammatory milieu (42–45), and because LTB₄ enhances NFκB activation and Myd88 expression (21, 27), we tested the possibility that LTB₄ promoted sterile inflammation in T1DM. Treating diabetic mice with a 5-LO inhibitor efficiently diminished LTB₄ production (Fig. 3A) and reduced the expression of Myd88 in macrophages to an amount similar to that in macrophages from nondiabetic mice (Fig. 3B). Moreover,
Insulin inhibits LTB₄-mediated Stat1 and Myd88 expression

Because T1DM is associated with hyperglycemia secondary to insulin insufficiency (46, 47), we sought to investigate the relative role of insulin in the regulation of the expression of Stat1 and Myd88 and the production of LTB₄. STZ-induced diabetes has been largely used as a model of insulin-dependent T1DM (31, 32). By contrast, mice with a spontaneous null mutation in the loci encoding the leptin receptor (db/db mice) spontaneously develop obesity, systemic insulin resistance, and hyperglycemia (48–50), which are hallmarks of T2DM. As expected, db/db mice developed hyperglycemia (fig. S1E), but, unlike STZ-treated mice, db/db mice were hyperinsulinemic (fig. S3A). Although type 2 diabetes is also associated with systemic low-grade inflammation (51, 52), the expression of Myd88 or Stat1 in macrophages from diabetic db/db mice did not substantially differ compared to that in nondiabetic controls (db/+ mice) from different strains that produce different amounts of insulin 2 months after the onset of diabetes (fig. S3, B to D).

Next, we investigated whether insulin treatment of T1DM mice restored LTB₄ production and Myd88 expression. Initially, we confirmed that insulin treatment restored blood glucose concentrations in T1DM mice to those measured in control mice (fig. 3H). We also confirmed that insulin decreased Alox5 expression in macrophages ex vivo and LTB₄ production in diabetic mice (Fig. 3I, I and J), which correlated with restoration of Myd88 expression to that of control mice (Fig. 3K). In vitro treatment of macrophages with insulin decreased expression of Stat1, Myd88, and Alox5 (fig. S4, A to C). To further investigate whether lack of insulin directly enhanced Alox5 expression, we used two different approaches. In the first approach, we treated wild-type mice with an insulin receptor antagonist, which increased Alox5 expression after 72 hours (fig. S5A). We used peritoneal macrophages for the second approach. Macrophages cultured in insulin and serum-free medium showed greater Alox5 expression than those cultured in serum containing medium or serum-free medium plus insulin (fig. S5B). To determine the molecular mechanisms underlying the enhancement of Alox5 expression during insulin deficiency, we used inhibitors of transcription factors and kinases known to enhance Alox5 expression (15). Our data show that extracellular signal–regulated kinase 1/2 (ERK1/2) inhibition, but not NFκB or PI3K (phosphatidylinositol 3-kinase), prevented increased Alox5 expression in insulin-free medium (fig. S5C). Together, these findings showed that lack of insulin accounts for the elevated Alox5 expression in insulin-free medium.
for the high basal amounts of LTB4 in macrophages in an ERK1/2-dependent manner.

**Enhanced LTB4 production accounts for enhanced susceptibility during polymicrobial sepsis in type 1 diabetic mice**

We postulated that LTB4 would induce a basal low-grade inflammatory state in T1DM mice, which would favor the formation of SIRS and increase mortality in these mice. We used two different models for assessing the consequences of LT inhibition. First, mice were treated with the 5-LO inhibitor AA-861 twice before the induction of polymicrobial sepsis, and cytokine production, bacterial load, and neutrophil recruitment were assessed. Then, mice were treated twice a day for 2 days after the onset of sepsis, and animal survival was determined (Fig. 4A). We also measured mortality after cecal ligation and puncture (CLP) in BLT1−/− diabetic and nondiabetic mice. T1DM mice succumbed to sepsis 24 hours after surgery, whereas ~60% of the control mice survived. Treatment of T1DM mice with the 5-LO inhibitor increased survival to 40%. Moreover, both control and diabetic BLT1−/− mice were protected from polymicrobial sepsis when 5-LO was inhibited (Fig. 4B). Serum concentrations of IL-1β and IL-10 were higher in T1DM mice than in septic control mice. Treatment with the 5-LO inhibitor decreased IL-1β production, enhanced IL-1RA production, and decreased IL-10 production in the blood 6 hours after sepsis; TNF-α was not detected (Fig. 4C). In the peritoneal cavity, the site of infection, 5-LO inhibition also decreased production of IL-1β and TNF-α in mice with T1DM (Fig. 4D). The increased survival of AA-861–treated mice correlated with a decrease in the numbers of neutrophils in the peritoneal cavity of diabetic mice (Fig. 4E). Furthermore, inhibition of 5-LO decreased bacterial load in the peritoneal cavity (Fig. 4F).

In aggregate, these findings show that a low-grade inflammatory response driven by the LTB4-BLT1 axis increases the cytokine storm, reduces bacterial load in the site of infection, and increases sepsis severity in mice with STZ-induced diabetes.

**DISCUSSION**

We have elucidated essential components of the molecular mechanisms involved in the generation of low-grade systemic inflammation in T1DM and its consequences in sepsis, a frequent morbidity associated with this disease. Low-grade inflammation is the main cause of morbidities associated with T1DM (53–55), which are closely associated with the production of IL-1β, IL-18, and IL-33 (13, 56). The receptors for these cytokines use MyD88 as a common adaptor to induce inflammatory responses (24). The increase in MyD88 and STAT-1 abundance in both STZ-induced diabetes (in C57BL/6 mice) and the genetically prone diabetic NOD mice, along with the increase in Myd88 expression in macrophages from different organs, underscores the importance of the diabetic milieu in the regulation of Myd88 expression. NFkβ activation is constitutively activated in different immune cells in both diabetic NOD and STZ diabetic mice (47, 57, 58), which favors production of inflammatory mediators and inflammatory diseases associated with diabetes. We speculate that enhanced NFkβ activation and generation of inflammatory mediators might be due to constitutive activation of TIR adaptors, such as MyD88, along with the generation of endogenous danger-associated molecular patterns that bind to different TLRs (24).

The production of LTB4 in T1DM has been previously shown. Boizel et al. (59) have shown that diabetic individuals constitutively produce LTB4, whereas Montero et al. (60) and Talahalli et al. (61) have demonstrated high amounts of LTB4 in the serum of STZ-treated diabetic mice. We found that both STZ-treated and NOD diabetic mice exhibited high basal levels of LTB4 in serum and in supernatants of macrophage cultures. We discovered that high basal LTB4 production correlated with enhanced expression of 5-LO in macrophages from two different murine models of T1DM. Although we studied the expression of Alox5 and LTB4 production in macrophages, other cells, such as neutrophils (15, 62), could also be involved in LTB4 production. However, the source of LTB4 in diabetics remains to be determined. Nonetheless, enhanced basal LTB4 production has implications for the inflammatory response that go beyond its effect on Myd88 abundance. LTB4 is a potent neutrophil chemoattractant and enhances the generation of reactive oxygen species, which cause tissue injury (15). LTB4 also stimulates NFkβ-dependent generation of proinflammatory cytokines (21, 63), which account for the basal low-grade inflammation observed in T1DM. LTB4 has been implicated as a major driver of inflammatory diseases, such as cardiovascular diseases and arthritis (16), and Myd88 depletion protects mice against these diseases (64). Therefore, elucidation of events related to the production of LTB4...
and expression of Myd88 are potentially relevant to identification of novel therapeutic targets in a myriad of diseases.

The lack of insulin production in type 1 diabetes distinguishes this disease from type 2 diabetes. Although T2DM is characterized by insulin resistance in metabolically active tissues (such as liver, skeletal muscle, and adipose tissue) and hyperinsulinemia, other tissues may remain insulin-responsive (65). Although the T2DM model used in our study relies on mice deficient in the leptin receptor and leptin activates macrophages (66), we think that the similar amounts of glycemia and hyperinsulinemia shown by these two different models of T2DM justify the use of these mice for comparison. Although T2DM is also characterized by low-grade inflammation, MyD88 abundance did not differ between diabetic db/db and nondiabetic db/+ mice. However, we cannot exclude that MyD88 abundance could be altered in different phases of T2DM. Adipocytes seem to be a source of LTB4 in T2DM (67), and thus, LTB4 could contribute to enhanced Myd88 expression in adipocytes in T2DM mice. LTB4 might also influence sterile inflammation by enhancing NFkB-mediated production of proinflammatory cytokines, such as TNF-α and IL-1β. Whether LTB4 controls chronic inflammatory responses in T2DM needs to be further explored.

Yano et al. have shown that insulin treatment reduces susceptibility to Staphylococcus aureus infection and restores host defense in diabetic db/db mice (68). In addition, insulin treatment restores the phagocytic capacity of macrophages and neutrophils in both type 1 and type 2 diabetic mice (68, 69). However, in our model, a lack of insulin, rather than hyperinsulinemia, plays a robust role in controlling sterile inflammation. We propose two mechanisms by which insulin can affect the host response. First, insulin may prevent secondary adverse effects of high blood glucose on immune function by correcting hyperglycemia. Second, insulin may directly influence macrophage activation. However, two of our findings suggest that hyperglycemia does not play a role in controlling LTB4 and Myd88 abundance in the T1DM model. First, mice in two models of T2DM exhibited hyperglycemia comparable to that shown by mice in our T1DM models. Second, treatment of wild-type naïve macrophages with insulin decreased Alox5 and Myd88 expression independently of the glucose concentrations.

Sterile inflammation is commonly associated with comorbidities associated with diabetes (5) and could contribute to the enhanced susceptibility to microbial sepsis of diabetics. Sepsis is characterized by the initial development of SIRS or cytokine storm, which may lead to septic shock and death (70). We confirmed that diabetic mice were more susceptible to polymicrobial sepsis, and showed that pharmacological 5-LO inhibition improved animal survival, decreased Myd88 expression, decreased cytokine production in the blood and peritoneal cavity, decreased neutrophil migration to the site of infection, and improved microbial clearance. Neutrophil recruitment to the site of infection is required for optimal microbial clearance during sepsis in murine models (71), and the antimicrobial effector function of neutrophils from diabetic mice and humans is lower than that of neutrophils from nondiabetic mice and healthy humans (68, 72). Therefore, we hypothesize that controlling the overproduction of LTB4 in diabetic mice not only reduces the initial systemic inflammation but also improves phagocyte functions and microbial clearance. Our data show that genetic deletion of BLT1 fully protected diabetic and nondiabetic mice against sepsis, which is in line with the results of Benjamin et al. These authors have shown that mice are protected from death after sepsis upon treatment with 5-LO activating protein inhibitor, but not with the BLT1 agonist CP105,696 (73). In contrast, Rios-Santos et al. have demonstrated that treatment with 5-LO activating protein inhibitor increased sepsis mortality (74). The reasons for these contradictory results are not known.

Nonetheless, our findings suggest that pharmacologic 5-LO inhibitors or BLT1 antagonists that are currently available or under development would be expected to reduce macrophage MyD88 abundance, reduce IL-1R activation, and thus reduce the chronic low-grade inflammatory response and associated morbidities in individuals with poorly controlled T1DM.

MATERIALS AND METHODS

Study design

For all experiments, the minimum sample size was determined to detect a difference between group means of two times the observed SE, with a power of 0.8 and a significance of 0.05, using the power and sample size calculator (www.statisticalsolutions.net/pss_calc.php). Thus, the calculated minimum sample sizes ranged from three to four depending on the experiment. The average sample size for mouse studies was five per group. All samples were randomized but not blinded.

Animals

Eight-week-old female 5-LO−/− [B6.129-Alox5tm1Flj; (75)], BLT1−/− [B6.129S4-Ltb4r1tm1Adl/J; (76)], and strain-matched wild-type C57BL/6 mice, NOD/ShiLtJ mice (77, 78), ICR/HAL mice, wild-type mice (C57BL/6J and C57BLKS/J), and mutant mice [db/db (C57BL/6J-m-lepr/db and C57BLKS/J-m-lepr/db), db/+ (C57BL/6J-m-leprdb/1)] (all from The Jackson Laboratory) were maintained according to National Institutes of Health guidelines for the use of experimental animals with the approval of the Indiana University Committees for the Use and Care of Animals.

Diabetes induction

T1DM was chemically induced by five sequential daily intraperitoneal injections of a freshly prepared solution of STZ (40 mg/kg) in 0.1 M citrate buffer (pH 4.5) (79). Blood glucose concentrations were measured 10 days after the last injection of STZ using Bayer CONTOUR glucometer and test strips (Bayer HealthCare LLC). Mice were considered diabetic when blood glucose concentrations reached >300 mg/dl on two consecutive days. The control group received five intraperitoneal injections of the vehicle. Serum insulin was measured using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem).

Macrophage isolation and stimulation

Macrophages were isolated from different anatomical sites. Peritoneal and alveolar macrophages were isolated and cultured as described (21, 27). Peritoneal macrophages were stimulated with LPS (100 ng/mL), LTB4 (100 nM), IL-1β (10 ng/mL), or insulin (2 mU/mL) for 24 hours followed by RNA or protein isolation and collection of cell supernatant for detection of LTB4 and cytokines. In another set of experiments, peritoneal cells were cultured in serum-free medium (macrophage-SFM Invitrogen) with or without insulin (10 U/mL) or Dulbecco’s modified Eagle’s medium (DMEM) plus 10% serum [which contains insulin (~10 U/ml)] for 24 hours. Macrophages cultured in the serum-free medium were incubated with the ERK1/2 inhibitor U0126 (10 µM), the PI3K inhibitor wortmannin (10 nM), and the NFκB inhibitor BAY117082 (10 µM), with doses previously tested (80, 81). Alternatively, C57BL/6 mice were injected intraperitoneally with the insulin receptor antagonist S961 (100 nM/kg) (82, 83) for 24 to 72 hours, and the peritoneal cells were harvested as described in the legends.

RNA isolation and real-time RT-PCR (qPCR)

Total RNA from cultured cells was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's
instructions. Complementary DNA (cDNA) was synthesized using a reverse transcription system (miScript II, Qiagen), and qPCR was performed with primers for Myd88, Stat1, Alox5, Lib4r1, Trif, Ticam, and β-actin (all from Integrated DNA Technologies) on the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) as previously described (21). Relative expression was calculated using the comparative threshold cycle (Ct) and calculated relative to control or wild type (ΔΔCt method).

**ChIP assay**

ChIP assays were performed with a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer’s protocol. Cells were fixed and cross-linked with 1% formaldehyde, and chromatin was digested with micrococcal nuclease and sonicated (UP100H; Hielsher) to obtain DNA fragments of about 150 to 900 base pairs. The resulting cross-linked chromatin preparation was subsequently enriched by immunoprecipitation with anti-c-Jun (1:100; Cell Signaling Technology) or anti–STAT-1 (1:50; clone H-300) antibodies. Normal rabbit immunoglobulin G (IgG) (1:100) and anti–histone H3 (1:50) antibodies (Cell Signaling Technology) were used as negative and positive controls, respectively. For each immunoprecipitation, 20 µg of cross-linked chromatin was diluted in ChIP buffer to a final volume of 0.5 ml, mixed with indicated antibodies, and incubated for 4 hours at room temperature with rotation. Immune complexes were captured using 30 µl of ChIP-Grade Protein G Magnetic Beads (Cell Signaling Technology) according to the manufacturer’s protocol. The chromatin was eluted from the beads by adding elution buffer and incubating at 65°C for 30 min followed by digestion with proteinase K for 2 hours at 65°C. Subsequently, DNA was purified using spin columns (Qiagen), and samples were subjected to real-time PCR using the primers for different promoter regions of Stat1 or Myd88 (tables S1 and S2).

**Immunoblotting**

Western blots were performed as previously described (20, 21). Protein samples were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and probed with commercially available primary antibodies against MyD88, STAT-1 (both 1:500; Abcam), or β-actin (1:10,000; Sigma-Aldrich). Densitometric analysis was performed as previously described (20, 21).

**Nitrite and cytokine measurements**

TNF-α, IL-10, IL-1β, and IL-1RA concentrations were measured using DuoSet ELISA (R&D Systems), and LTB4 was measured using an EIA (validated using spin columns (Qiagen), and samples were subjected to real-time PCR as previously described (21)). Relative expression was calculated using the comparative threshold cycle (Ct) and calculated relative to control or wild type (ΔΔCt method).

**Polymericial sepsis induction**

Sepsis was induced by CLP as previously described (64) with slight modifications. Briefly, mice were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) (intraperitoneally), and severe sepsis was induced by perforating their ceca with six superficial punctures using an 18-gauge needle. Sham mice received cecal ligation but no perforation of the cecum. One group of mice was treated with AA-861 (50 mg/kg, intraperitoneally; Cayman Chemical), 8 and 16 hours before and two times per day for 2 days after CLP surgery. Survival was monitored every 12 hours for 6 days after CLP surgery. Mice exhibiting signs of imminent death (inability to maintain upright position/ataxia/tremor and/or oral breathing) were euthanized. In a different experimental setting, mice were euthanized 6 hours after CLP surgery to investigate bacterial load, cytokine production, and leukocyte numbers.

**Bacterial load**

The peritoneal cavity was washed with phosphate-buffered saline 6 hours after CLP, and aliquots of serial dilutions were plated in Mueller-Hinton agar dishes as previously described (64).

**Leukocyte counts**

Leukocyte numbers were determined in the peritoneal cavity 6 hours after CLP using the Hemavet HV950FS System as previously described (64).

**Statistics**

Survival curves are expressed as percent survival and were analyzed by a log-rank (Mantel-Cox) test. Medians are shown for bacterial load results. Other results are presented as means ± SEM and were analyzed by analysis of variance (ANOVA) followed by Bonferroni analysis. Differences were considered significant when P < 0.05.

**SUPPLEMENTARY MATERIALS**

www.sciencesignaling.org/cgi/content/full/8/361/ra10/DC1

Fig. S1. Induction of chemically induced T1DM.

Fig. S2. Alveolar macrophages from T1DM mice show high basal expression of Myd88 and Stat1.

Fig. S3. Macrophages from diabetic db/db mice do not exhibit enhanced expression of Myd88 and Stat1.

Fig. S4. Exogenous insulin decreases Alox5, Myd88, and Stat1 expression in macrophages.

Fig. S5. Insulin deficiency enhances Alox5 expression in vivo and in vitro.

Table S1. Primers used for ChIP on the murine Myd88 promoter.

Table S2. Primers used for ChIP on the murine Stat1 promoter.

**REFERENCES AND NOTES**

RESEARCH ARTICLE


Funding: This work was supported by NIH grants (HL-103777 and R01HL124159-01 to C.H.S.; DK60581 and DK83583 to R.G.M.; DK099954 to C.E.-M.; DK100515 to D.L.M.; and T32 AI060519 to S.L.B.); Ralph W. and Grace M. Showalter Research Trust Fund (to C.H.S.); Veteran’s Affairs (VA) Merit Award I01BX001733 (to C.E.-M.); and gifts from the Sigma Beta Sorority, the Ball Brothers Foundation, and the George and Frances Ball Foundation (to C.E.-M.) and Fundação de Amparo a Pesquisa do Estado de São Paulo (to S.J. and L.R.F.). Author contributions: L.R.F. designed the research, performed the experiments, analyzed the data, and wrote the manuscript; S.L.B., S.W., Z.W., and D.L.M. performed the experiments and analyzed the data; C.E.-M., R.G.M., and S.J. designed the research and analyzed the data; and C.H.S. designed the research, supervised the work, analyzed the data, and wrote the paper. Competing interests: The authors declare that they have no competing interests.

Submitted 4 June 2014
Accepted 6 January 2015
Final Publication 27 January 2015
10.1126/scisignal.2005568

Leukotriene B₄–mediated sterile inflammation promotes susceptibility to sepsis in a mouse model of type 1 diabetes


Sci. Signal. 8 (361), ra10.
DOI: 10.1126/scisignal.2005568

Preventing sepsis in type 1 diabetics

Patients with type 1 diabetes have chronic systemic inflammation and are more prone to developing sepsis. Filgueiras et al. found that mice that are a model for type 1 diabetes had higher amounts of leukotriene B₄, a proinflammatory lipid, and of 5-lipoxygenase, the enzyme that produces leukotriene B₄. Mice with type 1 diabetes that were treated with an inhibitor of 5-lipoxygenase survived sepsis and had decreased markers of inflammation, suggesting that targeting 5-lipoxygenase to prevent the production of leukotriene B₄ could decrease the susceptibility of type 1 diabetic patients to sepsis.

REFERENCES

This article cites 83 articles, 26 of which you can access for free.

Use of this article is subject to the Terms of Service

Science Signaling (ISSN 1937-9145) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title Science Signaling is a registered trademark of AAAS.

Copyright © 2015, American Association for the Advancement of Science