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Leukotriene B₄-mediated sterile inflammation favors susceptibility to sepsis in murine type 1 diabetes

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Abstract

The chronic systemic inflammation in type I diabetes mellitus (T1DM), which is driven by signaling through the interleukin-1 (IL-1) 1 receptor (IL1R) and the adaptor protein myeloid differentiation factor 88 (MyD88), may be associated with the enhanced susceptibility of diabetics to systemic bacterial infection (sepsis). We hypothesized that low insulin concentrations trigger the enzyme 5-lipoxygenase (5-LO) to produce the lipid mediator leukotriene B₄ (LTB₄), serving as a trigger of systemic inflammation and increased susceptibility to polymicrobial sepsis in T1DM. In support of this hypothesis, we found that the abundance of MyD88 and its direct transcriptional regulator, STAT-1 were higher in peritoneal macrophages from two mouse models of T1DM compared to nondiabetic mice. Expression of *Alox5*, synthesis of LTB₄, and concentrations of the proinflammatory cytokine IL-1 β were also increased in peritoneal macrophages and serum from T1DM mice. Insulin treatment restored LTB₄ concentrations and *Myd88* and *Stat1* expression in T1DM mice. T1DM mice lacking *Alox5* or treated with a 5-LO inhibitor showed reduced *Myd88* and *Il1b* mRNA expression and increased IL-1 receptor antagonist concentration. The transcription factor cJun drove LTB₄-dependent transcription of *Stat1* in macrophages from T1DM mice. Compared to wild-type or untreated diabetic mice, T1DM mice lacking 5-LO or treated with a 5-LO inhibitor survived polymicrobial sepsis and showed reduced production of proinflammatory cytokines and decreased bacterial counts, suggesting that high LTB₄ concentrations contribute to enhanced susceptibility to sepsis in T1DM. These results uncover a role for LTB₄ in promoting sterile inflammation in diabetes and enhanced susceptibility to sepsis in T1DM.

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Keywords

eicosanoid; SIRS; sepsis; diabetes; leukotriene; 5-LO inhibitor; translational

Introduction

Imbalances between metabolism and the immune system have been linked with inflammatory diseases such as atherosclerosis, obesity, gout, and diabetes (1). Type I diabetes mellitus (T1DM) is an autoimmune disease characterized by destruction of pancreatic beta 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 (IL) -2, interferon (IFN)- γ , tumor necrosis factor (TNF) - α , and IL-1 β (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) and local infection, a condition frequently found in persons with diabetes (10–12).

Sepsis is a multifactorial disease involving an initial systemic uncontrolled production of inflammatory mediators, (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 5-lipoxygenase (5-LO)-derived bioactive lipid leukotriene B₄ (LTB₄) 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 G α i-mediated decreases in cyclic adenosine monophosphate (cAMP) concentrations enhances macrophage effector function (16–20). Inhibition of cAMP production increases pathogen recognition receptors (PRRs) activation of the pro-inflammatory transcription factor nuclear factor kappa B (NF κ B) (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 (LPS) from gram-negative bacteria. TLR family members and the IL-1 β receptor (IL-1 β R) share a conserved cytoplasmic Toll-IL-1R (TIR) domain that binds adaptor proteins, including myeloid differentiation factor 88 (MyD88) (24). MyD88 mediates signaling through all of the known TLRs except TLR3, but its importance for individual TLRs varies (24). Although it is similarly crucial for initiating

signaling responses to IL-1 β and other members of the IL- 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 as LTB₄. (25–27). We and others have shown that STAT-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 controlled by LTB₄, which enhances interleukin 1 receptor (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 enhanced IL-1 β production and decreased IL-1RA abundance, both of which favor IL1R 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.

Results

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

Since T1DM is accompanied by a constitutive low-grade inflammatory response, it seemed possible that T1DM mice would exhibit high MyD88 abundance, allowing the inflammatory response (4, 30–32). 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 (33, 34). Ten days after the induction of diabetes, mice exhibited similar body weights but higher glucose concentrations and lower insulin concentrations than control mice (Supplementary Fig. 1 A–D). *Myd88* and *Stat1* mRNA and protein abundance were higher in resident peritoneal macrophages from STZ-treated mice and mice with genetically induced T1DM (Non-Obese 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 (Supplementary Fig. 2). 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. 1 D), suggesting that the abundance of MyD88 and STAT-1 in macrophages is specifically altered in two different T1DM murine models.

We then determined whether macrophages from diabetic mice had increased responsiveness to receptors that exclusively utilize MyD88 to elicit macrophage activation (24, 35). We stimulated macrophages from T1DM and nondiabetic mice with the IL-1R1 agonist IL-1 β and TLR4 agonist LPS, which are dependent on MyD88 actions (24, 35). Macrophage activation was determined by the further enhancement of *Myd88* and *Stat1*, and the expression of the NF κ B-target inducible nitric oxide synthase (*Nos2*) and its product nitric oxide (NO) (36). Our results show that IL-1 β 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 mRNA encoding *Nos2* 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 IL1R1 responsiveness.

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

We have previously shown that LTB₄ enhances STAT-1 dependent *Myd88* expression in macrophages (21). Based on 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 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*. We found that *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. 2 C). Next, we sought to determine the roles of LTB₄ and BLT1 in controlling *Myd88* and *Stat1* expression in T1DM. Both *5-LO*^{-/-} and *BLT1*^{-/-} mice and their counterpart wild-type mice were treated or not with STZ to induce T1DM. As expected, in untreated mice, *5-LO*^{-/-} and *BLT1*^{-/-} macrophages exhibited lower *Myd88* and *Stat1* expression (Fig. 2 D and E). Neither *Myd88* nor *Stat1* expression was enhanced in macrophages from *5-LO*^{-/-} or *BLT1*^{-/-} T1DM mice (Fig. 2 D and E). Furthermore, no differences in either insulin or blood glucose abundance in STZ-treated *5-LO*^{-/-} or *BLT1*^{-/-} mice or diabetic mice treated with 5-LO inhibitor (Supplementary Fig. 1 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 investigated the molecular program through which the LTB₄-BLT1 pathway mediated *Stat1* expression. We determined whether the activity of the transcription factor cJun, which can activate *Stat1* expression (37), was stimulated by LTB₄ and whether cJun promoted *Stat1* transcription. Phosphorylation of Ser⁷³ in cJun (a phosphorylation event that is essential for its transcriptional activity, but not Ser⁶³ (38) was enhanced in macrophages from diabetic wild-type mice, but not in macrophages from *5-LO*^{-/-} diabetic mice, suggesting that leukotrienes promote cJun transcriptional activity (Fig. 2 F). 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. 2 G).

We have previously shown that silencing of *Stat1* abrogates LTB₄-induced *Myd88* expression in macrophages (21). Here, we sought to determine whether LTB₄ promoted STAT-1 binding to the *Myd88* promoter. Treatment of macrophages with LTB₄ increased binding of STAT-1 to *Myd88* promoter approximately 3-fold, as shown by ChIP assay (Fig. 2 H). We also tested two other promoter regions, but LTB₄-mediated STAT-1 binding to the *Myd88* promoter was specific to a single region (Fig. 2 H). 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

Since the majority of morbidities associated with type 1 and type 2 diabetes are due to the basal low-grade inflammatory milieu (39–42), and given the fact that 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 that efficiently diminished LTB₄ production (Fig. 3 A), macrophage *Myd88* expression was reduced to the same amounts found in macrophages from nondiabetic mice (Fig 3 B). Moreover, this treatment reduced the amounts of pro-inflammatory cytokines IL-1β and TNF-α in the serum (Fig. 3 C and E) and increased IL-1RA concentrations that was reduced in diabetic mice compared to nondiabetics (Fig. 3 D). This treatment had no effect on the glycemia of T1DM mice (supplementary Fig. 1 F). Production of these cytokines was ablated in both 5-LO^{-/-} and *BLT1*^{-/-} diabetic mice (Fig. 3 F and G), confirming that LTB₄ was the major 5-LO product involved in the induction of these cytokines. Together, our findings show that LTB₄ drives low-grade inflammation in the serum of type 1 diabetic mice by increasing IL-1β concentrations and promoting IL1R responsiveness by decreasing IL-1RA concentrations.

Insulin inhibits LTB₄-mediated *Stat1* and *Myd88* expression

Since T1DM is associated with hyperglycemia secondary to insulin insufficiency (43, 44), 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 (33, 34). By contrast, mice with a spontaneous null mutation in the leptin receptor loci (*db/db* mice) spontaneously develop obesity, systemic insulin resistance and hyperglycemia (45–47), which are hallmarks of T2DM. As expected, *db/db* mice develop hyperglycemia (Supplementary Fig. 1E), but, unlike STZ-treated mice, *db/db* mice are hyperinsulinemic (Supplementary Fig. 3 A). Although type 2 diabetes is also associated with systemic low-grade inflammation (48, 49), the expression of *Myd88* or *Stat1* in macrophages from diabetic *db/db* mice did not substantially differ compared to nondiabetic controls (*db/+* mice) from different strains that produce different amounts of insulin 2 months after the onset of diabetes (Supplementary Fig. 3 B–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 observed in control mice (Fig. 3 H). We also confirmed that insulin decreased *Alox5* expression in macrophages *ex-vivo* and LTB₄ production in diabetic mice (Fig. 3 I and J), which correlated with restoration of *Myd88* expression to that of control mice (Fig. 3 K). *In vitro* treatment of macrophages with insulin decreased expression of *Stat1*, *Myd88* and *Alox5* (Supplementary Fig. 4 A–C). To further investigate whether lack of insulin directly enhanced *Alox5* expression, we employed two different approaches: (1) wild-type mice were treated with an insulin receptor antagonist intraperitoneally for different days and, (2) peritoneal macrophages were cultured in serum containing media, and serum free media with or without insulin. Our results show that

insulin receptor antagonist increased *Alox5* expression after 72h of treatment (Supplementary Fig. 5 A). Furthermore, culture of macrophages in insulin and serum free media enhanced *Alox5* expression when compared to cells cultured in serum containing media or serum free media plus insulin (Supplementary Fig. 5 B). We then determined the molecular mechanisms involved in enhanced *Alox5* levels in insulin-free media. We inhibited the activation of transcription factors and kinases known to enhance *Alox5* expression (15) and also inhibited by insulin (10, 50–52) in both insulin- free and – containing media. Our data show that ERK 1/2 inhibition, but not NF κ B or PI3K inhibition prevented increased *Alox5* expression in insulin free media (Supplementary Fig. 5 C). Together, these findings showed that lack of insulin, rather than high insulin concentrations, accounts for the high basal amounts of LTB₄, which further increases *Myd88* and *Stat1*.

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 employed 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 was assessed. Then, mice were treated twice a day for two days after the onset of sepsis and animal survival was determined (Fig. 4 A). We also measured mortality after cecal ligation and puncture (CLP) in *BLT1*^{-/-} diabetic and nondiabetic mice. T1DM mice succumbed to sepsis 24 h after surgery, while ~ 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 with 5-LO inhibition (Fig. 4 B). Serum concentrations of IL-1 β and IL-10 were higher in T1DM mice than in septic control mice. Treatment with the 5-LO inhibitor decreased IL-1 β production, enhanced IL-1RA production, and decreased IL-10 production in the blood 6 h after sepsis; TNF- α was not detected (Fig. 4 C). In the peritoneal cavity, the site of infection, 5-LO inhibition also decreased production of IL-1 β and TNF- α in mice with T1DM (Fig. 4 D). 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. 4 E). Furthermore, inhibition of 5-LO decreased bacterial load in the peritoneal cavity (Fig. 4 F).

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. We found that: 1) macrophages from two mice models of T1DM exhibited increased abundance of MyD88 and STAT1; 2) T1DM mice exhibited higher production of LTB₄ levels in macrophages and serum, and

pharmacologic and genetic deletion of 5-LO and BLT1 prevented increased *Stat1* and *Myd88* expression in macrophages from diabetic mice; 3) Insulin deficiency, rather than hyperglycemia accounts for enhanced *Alox5* expression; 4) LTB₄ enhanced cJun mediated *Stat1* expression; 5) *In vivo* LT inhibition prevented production of the proinflammatory cytokines, and enhanced IL-1RA production; and 6) enhanced susceptibility to polymicrobial infection in diabetic mice was mediated by LTB₄/BLT1-dependent SIRS-mediated shock in diabetic animals.

Low-grade inflammation is the main cause of morbidities associated with T1DM (53–55), that are closely associated with the production of IL-1 β , IL-18, and IL-33 (13, 56). These receptors utilize MyD88 as a common adaptor to induce inflammatory responses (24). Therefore, studying the mechanisms involved in changes in MyD88 expression and actions might shed light on the intracellular programs involved in morbidities associated with chronic inflammation.

The fact that MyD88 and STAT-1 abundance was enhanced in both STZ-induced diabetes (in C57Bl/6 mice) and the genetically prone diabetic NOD mice (white background), along with the fact that *Myd88* expression was enhanced in macrophages from different organs underscores the importance of the diabetic milieu in the regulation of *Myd88* expression.

It has been shown that NF κ B activation is constitutively activated in different immune cells in both diabetic NOD and STZ-diabetic mice (44, 57, 58), which favors production of inflammatory mediators and inflammatory diseases associated with diabetes. We speculated that enhanced NF κ B 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).

Although the importance of MyD88 in inducing NF κ B activation is well established, the mechanisms underlying MyD88 expression in phagocytes are poorly understood. Here, we found that the LTB₄ was solely responsible for the enhanced MyD88 levels observed in macrophages from type 1 diabetic mice, and this activity of LTB₄ was dependent on STAT1 activation.

The production of LTB₄ in T1DM has been shown. Boizel et. al. (59) have shown that diabetic individuals exhibit constitutive levels of LTB₄, whereas Montero et al. and Tahalli et al. found high levels of LTB₄ in the serum of STZ-treated diabetic mice (60, 61). 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 further 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 expression. LTB₄ is a potent neutrophil chemoattractant and enhances the generation of reactive oxygen species, which cause tissue injury (15). LTB₄ also leads to activation of NF κ B-dependent generation of

proinflammatory cytokines (21, 63) that 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). Furthermore, MyD88 depletion protects mice against these diseases (29). 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 represents a major feature distinguishing type 1 from type 2 diabetes. Although T2DM is characterized by insulin resistance in metabolically active tissues (liver, skeletal muscle, and adipose tissue) and hyperinsulinemia, other tissues may remain insulin responsive (64). Even though the T2DM model used in our study relies on mice deficient in the leptin receptor and leptin is known to activate macrophage (65), the fact that these diabetic mice exhibit similar levels of glycemia and hyperinsulinemia justifies the use of these model for comparison. Whether MyD88 expression is altered in diet-induced obesity remains to be determined. While T2DM is also characterized by low-grade inflammation; we did not observe any changes in MyD88 abundance between diabetic *db/db* and nondiabetic *db/+* mice. However, we cannot exclude the fact that MyD88 abundance could be altered in different phases of T2DM. While we speculate that macrophages are the main source of LTB₄ in T1DM, adipocytes seem to be a source of LTB₄ in T2DM model (66). Thus, whether LTB₄ contributes to enhanced MyD88 expression in adipocytes from T2DM mice remains to be determined. Although in this work we did not investigate the participation of LTB₄ in T2DM, it might influence sterile inflammation by enhancing NFκB-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.

Yana et al. have shown that insulin treatment reduces susceptibility to *Staphylococcus aureus* infection and restores host defense in diabetic *db/db* mice (67). It has also been shown that insulin treatment restores the capacity of macrophages and neutrophils to phagocytosis in both type 1 and type 2 diabetic mice (51, 67). However, in our model, a lack of insulin rather than hyperinsulinemia plays a robust role in controlling sterile inflammation

The effect of insulin on the host response may be explained by two different mechanisms. First, insulin may prevent secondary adverse effects of high blood glucose on immune function by correcting hyperglycemia. Second, insulin may directly influences macrophage activation. We cannot rule out the possibility that hyperglycemia plays a role in the T1DM model by controlling LTB₄ and MyD88 abundance, although evidence suggests not: 1) Mice in two models of type 2 diabetes exhibit hyperglycemic levels comparable to those found in our T1DM models (Supplemental Fig 1E); 2) Treatment of wild-type naïve macrophages with insulin decreases *Alox5* and *Myd88* expression independent of the glucose levels (Supplemental Fig 3A–C); 3) *In vivo* AA-861 treatment does not decrease glycemia in diabetic mice (not shown) but decreases *Myd88* quantities. We further studied the relative role of insulin in the regulation of *Alox5* expression. These findings were accomplished in *in vivo* and *in vitro* studies where cells were cultured in the absence of insulin or mice treated with an insulin receptor antagonist. Different signaling programs control *Alox5* expression, including activation of MAPK p38 and ERK1/2, PI3K and NFκB. Our results suggest that

ERK1/2 activation is involved in enhanced *Alox5* expression in macrophages from T1DM mice.

Sterile inflammation is commonly associated with co-morbidities associated with diabetes (5) and could contribute to the enhanced susceptibility to microbial sepsis described in diabetics. Sepsis is characterized by the initial development of SIRS or cytokine storm, which may lead to septic shock and death (69). We confirmed that diabetic mice were more susceptible to polymicrobial sepsis, and that pharmacological 5-LO inhibition improved animal survival, decreased cytokine production in the blood and peritoneal cavity, decreased neutrophil migration to the site of infection, and improved microbial clearance. As evidence, we found that 5-LO inhibition prevented *in vivo* *Myd88* expression and improved the outcome of polymicrobial sepsis in mice. Neutrophil recruitment to the site of infection is required for optimal microbial clearance during sepsis in murine models (70). However, the role of neutrophils in microbial clearance of septic diabetic mice is unknown. It has been shown that neutrophils from diabetic mice and humans exhibits lower antimicrobial effector function than non diabetic mice and healthy humans (). 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. Although our current data clearly show that genetic deletion of BLT1 fully protected diabetic and nondiabetic mice against sepsis, contradictory results are reported.. While Rio-Santos et al found that treatment with 5-lipoxygenase activating protein inhibitor increased sepsis mortality (71), Benjamim et al found that this treatment prevented death after sepsis. The authors did not observe a protective effect in mice treated with the BLT1 antagonist CP105,696, although treatment of mice with the cysLT1 antagonist MK571 improved sepsis outcome (72). Our current data further show that genetic deletion of BLT1 fully protected diabetic and nondiabetic mice against sepsis.

In aggregate, our findings show that LTB₄ drives sterile inflammation in mice with T1DM. LTB₄ production is required for both the expression and actions of IL-1 β . LTB₄ increases IL-1 β release into serum, and influences IL-1 β actions by decreasing the levels of IL-1RA and increasing the expression of the IL-1R1 adaptor MyD88. These LTB₄-mediated changes in IL-1 β production and actions render mice more susceptible to septic shock, an event prevented by pharmacological inhibition of 5-LO or by genetic deletion of LTB₄.

Since chronic low grade inflammatory response is a major cause of morbidities associated with uncontrolled diabetes, our findings have direct translational importance, as pharmacologic 5-LO inhibitors or BLT1 antagonists that are currently available or under development would be expected to reduce macrophage MyD88 abundance and reduce IL1R activation in poorly controlled diabetic individuals.

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 level of 0.05, using the power and sample size calculator (<http://>

www.statisticalsolutions.net/pss_calc.php). On the basis of this, 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

8-week-old female *5-LO^{-/-}* (B6.129-*Alox5^{tm1Fur}*; (73)), *BLT1^{-/-}* (B6.129S4-*Ltb4r1^{tm1Adl}*/J; (74)), and strain-matched wild-type C57BL/6 mice, NOD/ShiLtJ mice (75, 76), ICR/HAL mice, wild-type mice (C57BL/6J and C57BLKS/J), mutant mice [db/db (C57BL/6J-m-leprdb/db and C57BLKS/J-m-leprdb/db), db/+ (C57BL/6J-m-leprdb/1) (all from The Jackson laboratories) were maintained according to NIH 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 (i.p.) injections of a freshly prepared solution of streptozotocin (40 mg/kg) in 0.1M citrate buffer (pH 4.5) (77). Blood glucose levels were measured 10 days after the last injection of STZ using Bayer Contour glucometer and test strips (Bayer HealthCare LLC, Pittsburgh, PA). Mice were considered diabetic when blood glucose levels reached > 300mg/dL on two consecutive days. The control group received 5 i.p. injections of the vehicle. Serum insulin was measured using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL).

Macrophage isolation and stimulation

Macrophages were isolated from different anatomical sites. Peritoneal and alveolar macrophages were isolated and cultured as described (21, 27). PMs were stimulated with LPS (100 ng/mL), LTB₄ (100 nM), IL-1 β (10 ng/mL), or insulin (2 mU/mL) for 24 h 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 media (macrophage-SFM Invitrogen) with or without 10 U/mL insulin or DMEM plus 10% serum (which contains ~ 10 U/mL of insulin) for 24h. Macrophages cultured in the serum-free media were incubated with ERK 1/2 inhibitor (U0126 10 μ M), PI3K inhibitor (wortmannin 10 nM) and NF κ B inhibitor (BAY117082, 10 μ M), with doses previously tested (78, 79). Alternatively, C57BL/6 mice were injected i.p. with the insulin receptor antagonist S961 (100 nM/kg,) (80, 81) for 24–72 h and the peritoneal cells were harvested at 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. cDNA was synthesized using a reverse transcription system (miScript II, Qiagen), and qPCR was performed using primers for *Myd88*, *Stat1*, *Alox5*, *Ltb4r1*, *Trif*, *Ticam*, and *beta actin* (all from Integrated DNA Technologies) on the CFX96 Real-Time PCR Detection System (Bio-

Rad Laboratories) as described (21). Relative expression was calculated using the comparative threshold cycle (Ct) and expressed relative to control or WT ($2^{-\Delta\Delta Ct}$ method).

ChIP assay

ChIP assays were performed using 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; Hielscher) to obtain DNA fragments of approximately 150–900-bp. The resulting cross-linked chromatin preparation was subsequently enriched by immunoprecipitation with anti-c-Jun (1:100; Cell Signaling) or anti-STAT-1 (1:50; clone H-300) antibodies. Normal rabbit 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 μ g 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 h at room temperature with rotation. Immune complexes were captured using 30 μ l 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 h at 65°C. Subsequently, DNA was purified using spin columns (Qiagen), and samples were subject to real time PCR using the primers for different promoter regions of *Stat1* or *Myd88* (Tables S1 and 2).

Immunoblotting

Western blots were performed as previously described (20, 21). Protein samples were resolved by 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 described previously (20, 21).

Nitrite and cytokine measurements

TNF-, IL-10, IL-1, and IL-1RA levels were measured using DuoSet ELISA (R&D Systems), and LTB₄ was measured using an EIA kit (Cayman Chemicals) following the manufacturers' protocols. Nitrite, the stable oxidized derivate of NO, was measured using the Griess reaction as described (82).

Polymicrobial sepsis induction

Sepsis was induced by CLP as previously described (29) with slight modifications. Briefly, mice were anesthetized with ketamine:xylazine (100 mg/kg:10 mg/kg, i.p.), and severe sepsis was induced by perforating their ceca with 6 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, i.p., Cayman Chemical), 8 and 16 hours before and 2 times/day for 2 days after CLP surgery (Fig. 6 A). 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 agonal 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 PBS 6 h after CLP, and aliquots of serial dilutions were plated in Mueller-Hinton agar dishes as described (29).

Leukocyte counts

Leukocyte numbers were determined in the peritoneal cavity 6 h after CLP using the Hemavet HV950FS System as described (29).

Statistics

Survival curves are expressed as percent survival and were analyzed by a Log-rank (Mantel-Cox) Test. Bacterial load results are expressed as median. Other results are expressed as mean $\pm$ SEM and were analyzed by ANOVA followed by Bonferroni analysis. Differences were considered significant when $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression and responsiveness to MyD88 and STAT1 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 determined by real time RT-PCR. (B) *STAT1*, *MyD88*, and β -actin protein expression in resident macrophages from control and STZ-diabetic mice determined by western. Relative *MyD88* and *STAT1* abundance was determined by densitometry (right); immunoblot results are representative of 3 independent experiments. Values for the 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 determined by real time PCR. **(D)** *Ticam* and *Trif* mRNA expression in resident peritoneal macrophages from NOD/ShiLtJ and control nondiabetic ICR/HAL mice determined by real time RT-PCR. **(E–J)** Resident peritoneal macrophages from STZ-treated and control (vehicle) mice were challenged with either IL-1 β **(E and F)** or LPS **(G–I)**, and cells or supernatant were harvested for determination of mRNA for *Myd88* **(E and G)**, *Stat1* **(H)**, *Nos2* **(I)**, or nitrite levels **(F and J)**. In all circumstances, data are expressed as mean $\pm$ SEM from at least three independent experiments with at least three mice per experimental group. * $p < 0.05$ compared to vehicle-treated mice or ICR/HAL mice. * $p < 0.05$ compared to vehicle control, # $p < 0.05$ compared to vehicle, LPS, or IL-1 β , and & $p < 0.05$ compared to non-stimulated macrophages from STZ-treated mice.

Figure 2. LTB $_4$ levels control transcriptional machinery involved in STAT1/MyD88 expression in macrophage from T1DM mice

(A) LTB $_4$ concentration in the supernatant of peritoneal macrophages from STZ-treated and vehicle-treated mice. (B) LTB $_4$ levels in the serum of STZ-treated or vehicle-treated mice or NOD/ShiLtJ or ICR/HAL mice were determined by ELISA (n=5 mice/group). (C) *Alox5* and *Ltb4R1* mRNA expression in macrophages from STZ-treated or vehicle-treated mice. (D) *Myd88* or (E) *Stat1* mRNA expression in diabetic and nondiabetic WT, 5-LO $^{-/-}$, or BLT1 $^{-/-}$ mice determined by real time PCR. (F) Phosphorylated (Ser 63 and Ser 73) and total

cJun immunoblotting of macrophages from diabetic and nondiabetic wildtype and 5-LO^{-/-} mice were determined by western blot; immunoblot results are representative of 2 independent experiments. Relative density of phosphorylated (Ser⁷³) and total cJun abundance was determined from densitometric analysis (right). Values for the wild-type non diabetic control group were set as 100%. **(G and H)** Formaldehyde-fixed chromatin from macrophages from wild-type and 5-LO^{-/-} mice (n=6 mice/group) treated or not with STZ were subjected to immunoprecipitation to enrich cJun **(G)** and STAT1 **(H)** complexes using specific antibodies. To assess the amount of transcription factor bound to three regions of the *Stat1* promoter or *Myd88*, quantitative real time PCR was performed with primers specific to portions of *Stat1* or *Myd88* promoter surrounding each transcription factor binding site pulled down by ChIP. In all circumstances, data are expressed as mean ± SEM from at least three independent experiments with at least three mice per experimental group *p > 0.05 compared to vehicle-treated mice or ICR/HAL mice. &p<0.05 compared to vehicle control or ICR/HAL mice, #p<0.05 compared to naïve macrophage from STZ-treated mice.

Figure 3. 5-LO inhibition abolishes systemic inflammation in T1DM mice

C57BL/6 mice with STZ-induced T1DM were treated or not with the 5-LO inhibitor AA-861 every 24 h for two days, and the concentration of LTB₄ in the serum (A; n=5 mice/group), expression of *Myd88* in macrophages (B) and the production of IL-1 β (C), IL-1RA (D), and TNF- α (E) in the serum was determined by ELISA (n=5 mice/group). Serum from C57BL/6, 5-LO^{-/-} and BLT1^{-/-} T1DM were harvested and the levels of IL-1 β (F) and TNF- α (G) were determined by ELISA (n=5 mice/group). (H) glycemia (n=7 mice/group), (I) *Alox5* mRNA expression, (J) serum LTB₄ (n=7 mice/group), and *Myd88* mRNA (K) were determined in macrophages from T1DM mice treated with insulin every 12 h for two days. In all circumstances, data are expressed as mean $\pm$ SEM from at least three independent experiments. *p < 0.05 compared to vehicle-treated mice; #p < 0.05 compared to wild-type STZ STZ-treated mice only.

Figure 4. Elevated basal LTB₄ levels drive susceptibility to sepsis in T1DM

(A) Treatment protocol for LT inhibition for the determination of parameters involved in sepsis severity and determination of animal survival after T1DM. (B) Survival rates of T1DM and nondiabetic C57BL/6 mice were treated or not with AA-861 as in A or diabetic and nondiabetic *BLT1*^{-/-} mice followed by moderate CLP-induced sepsis (n=7–11 mice/group). Diabetic C57BL/6 mice were pretreated or not as in (A) and subjected to CLP. (C and D) Concentration of IL-1 β , IL-1RA, or IL-10 in the serum (C) or peritoneal lavage fluid (D) were determined by ELISA after CLP (n=7 mice/group). (E) Neutrophil numbers in the peritoneal cavity were determined using Hemavet (n=7 mice/group). (F) CFU in peritoneal cavity 6 h after CLP in diabetic and nondiabetic mice treated as in (A), (n=5 mice/group). (G) Proposed model of exaggerated LTB₄ production and enhanced MyD88 expression in T1DM. **p* < 0.05 relative to the sham group or nondiabetic CLP compared to AA-861 treatment or *BLT1*^{-/-} mice. PC, peritoneal cavity.