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IL-1β-Mediated Innate Immunity Is Amplified in the db/db Mouse Model of Type 2 Diabetes

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Keith W. Kelley,* Robert Dantzer,‡ Rodney W. Johnson,* and Gregory G. Freund2*†

Chronic inflammation, characterized by elevated circulating levels of proinflammatory markers, including IL-1β, appears to play a critical role in the pathogenesis of type 2 diabetes and its associated complications (1–5). Importantly, dysregulated and/or excessive inflammation can exacerbate pre-existent disease states (6–8) and profoundly depress mood and the feeling of well-being (9, 10). However, the possible association between peripheral inflammation and central inflammation has not been tested in animal models of type 2 diabetes, despite the obvious clinical implications. Initial host defense against infectious pathogens is mediated by the innate immune system requiring activation of inflammation-dependent cytokine networks (11). Appropriate regulation of these networks is essential to resolving infection (12), and APCs, especially macrophages, are the principal elaborators of this early cytokine response. Interestingly, patients with type 2 diabetes suffer from delayed wound healing and higher infection-related mortality rates than the general population (13), indicating a link between dysregulated inflammation and well-being. Activated macrophages secrete proinflammatory cytokines that direct local inflammation, initiate the acute phase response, communicate immune status to the CNS, and cause the brain-based host response to infection known as sickness behavior (14). Importantly, this innate immune response induces host behavioral adaptation, which is designed to facilitate recovery from illness and minimize transmission of infectious agents (15). Sick individuals experience weakness, malaise, fatigue, and an inability to concentrate. In addition, they are lethargic and show little interest in their surroundings. These pathogen-induced behavioral aspects of the innate immune response represent a highly organized change in motivational priorities (15) that offer researchers the opportunity to measure functional consequences of inflammation on the brain.

We (16, 17), and others (18), have shown that i.p. administration of LPS, the active component of endotoxin from Gram-negative bacteria, induces brain-based innate immunity. Following i.p. LPS, microglial synthesis and secretion of IL-1β is increased in the choroid plexus and in circumventricular organs where it directly activates neurons of the basolateral amygdala and the area postrema (19). LPS-induced behavioral adaptation is absent in IL-1 converting enzyme knockout mice (20). Furthermore, the behavioral aspects of the innate immune response initiated by either peripherally or centrally administered IL-1β are blocked by intracerebroventricular (ICV)3 administration of antisense oligonucleotides for the type 1 IL-1 receptor (IL-1R1) (21), passive immunoneutralization with mAbs directed at IL-1R1 (21) or by IL-1R1 knockout in mice (22). IL-1R1 knockout mice are also resistant to Gram-negative infection, experiencing a 43% reduction in mortality at 7 days (23).

The biological activity of IL-1β is, in part, counterregulated through IL-1R antagonist (IL-1ra) and a second receptor isoform, IL-1R2. IL-1ra binds the active receptor (IL-1R1) with high affinity but does not initiate the intracellular signaling cascade (24). IL-1R2 acts as a competitive inhibitor that binds and sequesters IL-1β but lacks a functional intracellular Toll-IL-1 receptor domain (25). After LPS or peripheral IL-1β administration, peak brain expression of IL-1ra and IL-1R2 occurs after that of IL-1β

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3 Abbreviations used in this paper: ICV, intracerebroventricular; IL-1ra, IL-1R antagonist.
Importantly, mice overexpressing IL-1ra are protected from lethal endotoxemia, while mice deficient in IL-1ra show increased susceptibility to endotoxin-induced lethality (28). We have shown that central administration of rIL-1ra inhibits sickness behavior induced by IL-1β (29). In addition, peripheral administration of IL-1ra attenuates the LPS-induced sickness response and improves recovery time from LPS-induced fever (30). Finally, immunoneutralization of IL-1R2 potentiates IL-1β-induced anorexia (31). Because chronic inflammation is associated with type 2 diabetes, and IL-1 is known to be a critical cytokine mediator of inflammation, we sought to demonstrate that innate immunity was amplified in db/db mice through a mechanism dependent on diabetes-associated loss of IL-1β counterregulation. Importantly, our results also show for the first time that the defective inflammatory response to LPS that occurs peripherally in db/db mice has its symmetrical counterpart in the brain.

Materials and Methods

Materials

All reagents and chemicals were purchased from Sigma-Aldrich except as noted below. FCS (0.05% bovine serum albumin, 0.48 mg/ml endotoxin) and recombinant murine IL-1β were purchased from Atlanta Biologicals. Bio-Rad protein reagent was purchased from Bio-Rad. Primer pairs were purchased from Qiagen. Sybr Green PCR master mix and Microamp optical 96-well reaction plates were purchased from Applied Biosystems. TRIZol was purchased from Invitrogen Life Technologies. SuperScript III RNase H reverse transcriptase, dNTP mix, and oligo dT primers were purchased from Invitrogen Life Technologies. RNAsin RNase inhibitor was purchased from Promega. One Touch Ultra glaciometer and glucose strips were purchased from Johnson & Johnson. Sensitive rat insulin radioimmunoassay kit was purchased from Linco Research. Mouse cannulas and cyanoacrylate gel adhesive were purchased from Plastics One. Murine IL-1β ELISA reagents, IL-1β polyclonal Ab (PM4254B), biotin-labeled IL-1β mAb (MM4255B), HRP-conjugated streptavidin, TMB substrate solution (n301), and mouse IL-1β ELISA standard (SMI11B) were purchased from Endogen. IL-1ra ELISA standard, IL-1ra polyclonal Ab, and biotinylated IL-1ra mAb were purchased from R&D Systems. Maxisorp coated 96-well ELISA plates were purchased from Nalg Nunc International.

Animals

All animal care and use was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NRC) as we described (32). Briefly, 8- to 12-week-old db/+ or db/db mice as we described (32). Briefly, blood glucose levels were measured using a One Touch Ultra glucometer per the manufacturer’s instructions. For random and fasting blood glucose measurements, blood was collected at 9:00 a.m. from mice fed ad libitum or fasted overnight, respectively. Serum insulin levels were measured by RIA according to the manufacturer’s instructions. For random serum and fasting insulin levels, blood was collected as above and serum was fractionated by centrifuging whole blood for 10 min at 5000 × g.

Sickness behavior

The sickness behavior response was measured using a social exploration test we have described (16). Briefly, mice were i.p. injected with LPS (Escherichia coli, 0127:B8), recombinant murine IL-1β, or vehicle in a volume of 0.25 ml at the indicated concentrations. At the times indicated, a 3- to 4-week-old novel conspecific juvenile mouse was placed in the home cage of the adult subject mouse for 10 min. The interaction between the subordinate juvenile and juvenile during this time period was video recorded. Duration of subject mouse-initiated exploratory behavior of the juvenile was determined from the video records. To control for mouse-to-mouse variability in baseline activity and allow comparison of relative changes in exploration levels, a preinjection (0 h) measurement was used as an internal control for each mouse, and the results are expressed as percentages of the baseline measurement and shown as means ± SEM.

RNA isolation and reverse transcription

Total RNA from spleen or whole brain samples was extracted into TRIZol. Reverse transcription was performed using oligo dT primers added to either 1 μg of spleen or 3 μg of brain RNA. The oligo dT primer/RNA mix was then inactivated at 70°C for 10 min and chilled on ice. Reverse transcription was performed at 42°C for 90 min in a reaction buffer containing 10 mMol/L DTT, 0.5 mMol/L (each) dNTP, 200 U SuperScript III, 93.75 mMol/L KCl, 3.75 mMol/L MgCl2, 62.5 mMol/L Tris-HCl, pH 8.3. The reaction was terminated by heat (70°C for 15 min). To minimize interassay variation all RNA samples from a single experimental group were reverse transcribed simultaneously.

Real-time PCR

Real-time PCR was performed as previously described (33). In brief, Primer Express software (Applied Biosystems) was used to design appropriate primers pairs. The primer sequences used were: β-actin forward- GGCCTTTTGTACCTAGGATG; β-actin reverse-GGATGTTGGCTC AACCA; IL-1β forward-CTGGTGGTCTCGGGTGACCC; IL-1β reverse- CAGCTCATATGGTGCCAGCA; IL-1βra forward-TTATAGCT CACCCATGCTCTCA; IL-1βra reverse- GCATCTGGAGGCCTTTC; IL-1R2 forward- GCCTCAGTCTCTCTACTCIGA; IL-1R2 reverse- CTTCACTGTCGGCAACTACGT. Real-time RT-PCR was performed on the ABI Prism 7700 (Applied Biosystems) using Sybr Green PCR Master Mix following the manufacturer’s instructions. To normalize gene expression, a parallel amplification of endogenous and target genes was also performed. Amplifications without reverse transcription or template were included as negative controls. Relative quantitative evaluation of the amplification products was performed by comparing threshold cycle (ΔCt), as previously described (Applied Biosystems user bulletin no. 2).

ELISA

Polyclonal anti-mouse IL-1β Ab at 2 μg/ml or polyclonal anti-mouse IL-1ra Ab at 1 μg/ml was absorbed to 96-well microtiter plates overnight at 25°C. Wells were then blocked with 4% BSA/PBS for 1 h and washed with 0.2% Tween 20, 50 mMol/L Tris, pH 8.0. Standards and experimental samples were incubated at 25°C for 2 h in the presence of biotin labeled monoclonal anti-mouse IL-1β or anti-mouse IL-1ra Ab (250 ng/ml). Wells were washed, and HRP-conjugated streptavidin (78 ng/ml) was added for 30 min at 25°C. After washing, 3,3’,5,5’-tetramethylbenzidine substrate solution was added for 30 min at 25°C. HRP/substrate reaction was terminated with an equal volume of 0.18 M H2SO4 and absorbance measured on an OPTImax tunable microplate reader (Molecular Devices) at 450–550 nm. IL-1β or IL-1ra concentrations were determined by reference to the standard curve and normalized to the control in the same reaction plate.

Peritoneal macrophage isolation

As we have described (32), mice were sacrificed by CO2 asphyxiation and peritoneal cells were collected by peritoneal lavage using two 5-ml washes of ice-cold growth medium (RPMI 1640 supplemented with 10% FCS, 2 g/L sodium bicarbonate, 110 mg/L sodium pyruvate, 100 mg/L streptomycin, and 10 mMol/L HEPES, pH 7.4). Macrophages were then isolated from the lavage fluid by adherence to plastic using the following procedure. Lavage cells were pelleted and resuspended in 10 ml of hypertonic RBC lysis buffer (142 mMol/L NaCl, 1 mM KHCO3, 118 mMol/L NaEDTA, pH 7.4) at room temperature for 10 min then mixed 1:1 with growth medium, pelleted, and resuspended at 37°C. Cells were plated at 5 × 105 cells/ml and after 30 min plates were washed twice to remove nonadherent cells, resulting in >80% pure macrophages, confirmed by CD11b staining and morphology (34).

IVC administration of IL-1β

As we have described (35), mice were anesthetized with a sodium ketamine hydrochloride/xylazine hydrochloride solution (80 mg/ml/12 mg/ml, ketamine/xylazine) at 1.5 mg/kg body weight and placed in a Kopf stereotaxic device (David Kopf Instruments). A sterile, 28 gauge mouse brain infusion cannula was stereotaxically implanted into the lateral ventricle at the coordinates 0.6 mm posterior/1.5 mm lateral to the bregma and 2.5 mm ventral from the surface of the skull. Cannulae were fixed to the skull with cyanoacrylate gel adhesive. Mice were allowed to recover for 5 days before experimentation. For experimentation, 2 μl of recombinant murine IL-1β (1 ng/μl) or PBS was infused into the lateral ventricle at a rate of 1 μl/minute.

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Statistical analysis
Data are presented as mean ± SE. Where indicated, experimental data was analyzed by the Student’s t test for comparison of means, or by two-factor ANOVA using Microsoft Excel. Statistical significance was denoted at \( p < 0.05 \).

Results

Intensity of the innate immune response to LPS is increased in type 2 diabetic mice

We have previously shown that a hallmark of the innate immune response to Gram-negative bacterial pathogens is sickness behavior mediated by IL-1β (14, 22, 26, 29, 35, 36). To determine whether innate immunity was perturbed in the db/db mouse model of type 2 diabetes, sickness behavior was examined in diabetic homozygous (db/db) and nondiabetic heterozygous (db/+ ) mice (Table I). Fig. 1 shows that when LPS was administered i.p. (100 μg/kg/mouse) to db/db mice social exploration was 46 ± 5.5, 30.4 ± 2.6, 37.8 ± 8.4, and 46.9 ± 4.0% of basal exploration at 2, 4, 8, and 12 h after i.p. injection, respectively. By 24 h, sickness behavior had resolved 89.5 ± 4.8%. This reduction in social exploration in db/db mice was significantly greater than that observed in similarly treated db/+ mice at the same time points (61.0 ± 4.1, 63.4 ± 5.5, 82.5 ± 5.8, 80.9 ± 6.5, and 100.5 ± 6.2% of basal exploration). Importantly, PBS sham-treated db/db and db/+ mice showed no reduction in exploratory behavior at the times examined. In addition, there were no baseline behavioral differences between untreated db/+ and db/db mice (data not shown). These results indicate that type 2 diabetic mice have an augmented innate immune response to LPS administered at a body-weight-adjusted dose.

Recovery from sickness is delayed in type 2 diabetic mice

As shown in Table I, db/db mouse body weight is one-third greater than that of nondiabetic db/+ mice. Therefore, in Fig. 1, db/db mice received one-third more LPS than db/+ mice. To determine how a fixed dose of LPS impacted innate immunity, sickness behavior was examined in diabetic db/db and nondiabetic db/+ mice both receiving 5 μg/mouse LPS administered i.p. Through dose-effect studies, we determined that 5 μg/mouse LPS per mouse resulted in the maximum depression in behavior while maintaining a full return to baseline by 24 h (data not shown). Fig. 2 demonstrates that maximum reduction in exploratory behavior at 2 h was similar in LPS-treated db/db (92.6 ± 3.3) and db/+ (83.1 ± 7.1) mice. However, db/db mice recovered significantly more slowly from i.p. LPS than LPS-treated db/+ mice (22.2 ± 0.8 vs 40.6 ± 5.1%, 36.8 ± 4.3 vs 59.0 ± 1.8%, and 57.3 ± 2.5 vs 71.8 ± 3.5% of baseline exploration at 4, 8, and 12 h, respectively). By 24 h, both db/db and db/+ mice were fully recovered. As in Fig. 1, PBS sham-treated db/db and db/+ mice showed no reduction in exploratory behavior at the times examined. These results indicate that type 2 diabetic mice have impaired recovery from LPS-induced initiation of the innate immune response.

LPS-induced IL-1β secretion from macrophages is increased in diabetic mice

We have shown that IL-1β produced by peritoneal macrophages in response to LPS is necessary to innate immune-based sickness behavior during peritoneal inflammation (37). To determine whether macrophages from diabetic mice elaborate more IL-1β in response to LPS, resident peritoneal macrophages were isolated from untreated db/db and db/+ mice and stimulated with LPS ex vivo. Fig. 3a shows that resident macrophages from db/db mice elaborate significantly more IL-1β in response to LPS at all LPS concentrations examined (1 ng LPS, 44.2 ± 7.9 vs 16.9 ± 3.9 pg IL-1β/mg total protein; 10 ng LPS, 50.3 ± 4.3 vs 32.9 ± 5.1 pg IL-1β/mg total protein; 100 ng LPS, 80.1 ± 7.8 vs 46.2 ± 3.6 pg IL-1β/mg total protein). To confirm that these findings were biologically relevant, we next examined IL-1β levels in peritoneal fluid from db/db and db/+ mice administered 5 μg/mouse LPS i.p. Fig. 3b demonstrates that peritoneal IL-1β concentrations were increased in db/db mice compared with db/+ mice 2.3-fold (1498.9 ± 192.9 vs 650.4 ± 75.5 pg IL-1β/ml) at 2 h after LPS administration, respectively. Importantly, endogenous secretion of IL-1ra is necessary to counter regulation of IL-1β-mediated inflammation (28). To determine whether IL-1ra was perturbed in db/db mice, peritoneal fluid was examined for IL-1ra as in Fig. 3b. Fig. 3c shows that basal peritoneal levels of IL-1ra were markedly reduced in db/db mice compared with db/+ mice (54.4 ± 39.2 vs 779.8 ± 283.5 pg/ml), and that LPS-induced IL-1ra up-regulation in db/db mice was less than half that of db/+ mice (1203.3 ±

Table I. db/db mice have elevated body weight, blood glucose, and serum insulin levels

<table>
<thead>
<tr>
<th></th>
<th>db/+</th>
<th>db/db</th>
</tr>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.56 ± 0.55</td>
<td>36.72 ± 0.57*</td>
</tr>
<tr>
<td>FBG (mg/dl)</td>
<td>115.2 ± 19.08</td>
<td>400.4 ± 15.35*</td>
</tr>
<tr>
<td>RBG (mg/dl)</td>
<td>169.8 ± 12.23</td>
<td>423.0 ± 17.84*</td>
</tr>
<tr>
<td>FSI (ng/ml)</td>
<td>1.34 ± 0.05</td>
<td>2.36 ± 0.05*</td>
</tr>
<tr>
<td>RSI (ng/ml)</td>
<td>2.16 ± 0.08</td>
<td>5.41 ± 0.09*</td>
</tr>
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* Fasting blood glucose (FBG), random blood glucose (RBG), fasting serum insulin (FSI), and random serum insulin (RSI). Results represent the average of five mice ± SEM.

* p < 0.05.

FIGURE 1. Intensity of the innate immune response to LPS is increased in type 2 diabetic mice. db/+ or db/db mice were injected i.p. with a fixed dose of LPS (5 μg/mouse) or an equal volume of PBS. Social exploration was measured immediately before the injection and 2, 4, 8, 12, and 24 h postinjection. Results are expressed as percentages of the baseline measurement and shown as means ± SEM; n = 5. (*, p < 0.05 vs db/+ LPS)

FIGURE 2. Recovery from sickness is delayed in type 2 diabetic mice. db/+ or db/db mice were injected i.p. with a fixed dose of LPS (5 μg/mouse) or an equal volume of PBS. Social exploration was measured immediately before the injection and 2, 4, 8, 12, and 24 h postinjection. Results are expressed as percentages of the baseline measurement and shown as means ± SEM; n = 5. (*, p < 0.05 vs db/+ LPS)
PBS sham treatment had no effect. These results indicate that key
mRNA expression was increased 2.5-fold, respectively, while
3.5-fold, respectively. In
mRNA expression was increased 9.7-fold, 27.7, 52.9, and 66.6, respectively.

We (29) and others (31) have shown that IL-1 antagonists like IL-1ra and IL-1R2 counterregulate sickness behavior during the innate immune response. To further examine IL-1β inhibition, real-time RT-PCR was performed on total splenic RNA from 
and 
were injected i.p. with 
PBS. At 0 and 2 h, i.p. IL-1β or IL-1ra protein levels were measured by ELISA. Data represent means ± SEM; n = 4. (*, p < 0.05)

LPS-induced IL-1ra and IL-1R2 mRNA expression is blunted in 

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As shown in Figs. 3 and 4, perturbation of the IL-1 axis in response to LPS appears to be responsible for the enhanced innate immunity seen in 

These results indicate that type 2 diabetic mice have an amplified innate immune response to IL-1β.

Direct administration of IL-1β recapitulates the LPS effects in diabetic mice

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Negative regulators of IL-1β function fail to up-regulate during an LPS-induced innate immune response in type 2 diabetic mice.

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Brain-based responsivity to IL-1β is augmented in the db/db mouse

As shown in Fig. 4, the IL-1β antagonists IL-1ra and IL-1R2 are not up-regulated in the spleen in response to peripheral administration of LPS. To determine whether the same findings were present in the brain, real-time RT-PCR was performed on total cerebral RNA from db/db and db/+ mice administered 5 μg/mouse LPS i.p. Fig. 6a demonstrates that IL-1ra mRNA expression was not up-regulated in db/db mice exposed to LPS. At 2, 4, and 8 h, db/db mouse IL-1ra mRNA expression was no different from PBS injected mice. In db/+ mice, IL-1ra mRNA expression was increased 1.4 ± 0.3-, 2.6 ± 0.5-, and 2.9 ± 0.7-fold at the same time points. PBS sham treatment had no effect. Examination of LPS-induced IL-1R2 mRNA expression in db/db mice showed a similar blunting. At 2, 4, and 8 h, db/db mouse IL-1R2 mRNA expression was not increased in response to i.p. LPS, while db/+ mouse expression was increased 1.6 ± 0.3-, 2.9 ± 0.5-, and 3.3 ± 0.6-fold (Fig. 6b). As above, PBS sham treatment had no impact. Finally, to determine whether brain-based responsivity to IL-1β was elevated in db/db mice, murine IL-1β (2 ng/mouse) was infused into the lateral ventricle of the brain. Fig. 6c demonstrates that maximum reduction in exploratory behavior was similar in IL-1β-treated db/db (93.4 ± 4.3%) and db/+ (89.6 ± 2.5%) mice. However, db/db mice recovered significantly more slowly than did db/+ mice (17.3 ± 4.6 vs 41.0 ± 5.8%, 26.9 ± 3.4 vs 61.7 ± 8.5%, and 49.2 ± 5.2 vs 76.4 ± 1.4% baseline exploration at, 4, 6, and 8 h, respectively). As in all the behavior experiments PBS sham treatment had no effect. Taken together, these data indicate that endogenous inhibitors of IL-1β function fail to up-regulate in the db/db mouse brain in response to LPS and that brain-based recovery from IL-1β stimulation is delayed.

Discussion

There is accumulating evidence that systemic activation of the innate immune system characterized by chronic low-grade inflammation is involved in the pathogenesis of both type 2 diabetes and many of its associated complications (1–5). Several studies have shown that circulating markers of inflammation (IL-1β, TNF-α, and IL-6) (4, 5) and acute phase reactants, like C-reactive protein, serum amyloid A, and sialic acid (1–3) are elevated in individuals with type 2 diabetes and are strong predictors of the development of type 2 diabetes in otherwise healthy individuals. In addition to mediating local inflammation and the acute phase response, the innate immune system communicates immune status to the CNS to orchestrate the sickness behavior response where an organism’s motivational priorities temporarily change to more efficiently fight infection (15). It is well established, in animal models and individuals with type 2 diabetes, that the wound healing process takes significantly longer than in nondiabetic control subjects (38). Furthermore, patients with type 2 diabetes have higher infection-related mortality rates than individuals without diabetes (13). This, coupled with chronically elevated levels of circulating inflammatory markers, strongly suggests that type 2 diabetes impairs the ability of the innate immune system to appropriately regulate the inflammatory response. However, until now it has not been recognized that the brain-based innate immune response is augmented in type 2 diabetes.

LPS-induced social withdrawal is a classic feature of the innate immune response that is routinely used as a quantitative measure in rodents because the large decline in activity is reliable and easily measured (26). Fig. 1 shows that LPS-induced sickness behavior was significantly augmented in db/db mice as compared with nondiabetic control mice. This finding demonstrates that the heightened brain-based innate immune response we found in type 1 diabetic NOD mice (39) occurs in type 2 diabetes. The importance of this observation is that altered central innate immunity now appears to be a common feature of all types of diabetes. Supporting this conclusion is that augmented neuroimmunity may also be seen in diabetes predisposing conditions like obesity because obese (fa/ff) Zucker rats compared with lean (FA/FA) rats display elevated fever and hypothalamic-pituitary-adrenal axis activity in response to LPS (40).

db/db mice, as Fig. 1 demonstrates, were more responsive to i.p. LPS both in terms of magnitude and in duration of sickness behavior. Although a dose of 100 μg/kg LPS (Fig. 1) is commonly used in the study of neuroimmunity in rodents, under these conditions db/db mice received a significantly larger dose of LPS because of their obesity-associated increase in body weight (Table 1). Clearance of LPS from the peritoneal cavity occurs primarily hematogenously via the portal vein and by the peritoneal lymphatic system (41). Importantly, only 85% of 125I-labeled LPS in the peritoneum is cleared by 6 h after i.p. LPS administration (42). Because abdominally based inflammation uses the vagus nerve to communicate with the brain, locally elevated LPS levels in the peritoneum (due to a weight based strategy of LPS administration) might artificially heighten sickness behavior in obese mice vs thin mice. To prevent this problem, we examined db/db mouse LPS-dependent neuroimmunity using a fixed dose of LPS (Fig. 2). Interestingly, these data indicated that maximum sickness behavior...
was similar in diabetic and nondiabetic animals but that recovery from sickness was significantly delayed. The importance of these studies is that they are the first to show in a quantitative fashion that type 2 diabetes is associated with a decreased ability to recover from inflammatory processes that activate the brain innate immune system.

A logical mechanism for the increased sickness behavior observed in db/db mice (Figs. 1 and 2) would be that macrophages from db/db mice elaborate more IL-1β in response to LPS than nondiabetic mice. In Fig. 3 we found that macrophages from db/db mice produced more IL-1β after LPS stimulation and that the peritoneal fluid of db/db mice exposed to a fixed dose of LPS had a peak increase in IL-1β concentration more than double that of nondiabetic mice exposed to LPS. Interestingly, while type 2 diabetes is widely believed to be associated with a state of perturbed innate immunity, the exact effect of diabetes on macrophage function is less clear. Naguib et al. (43) indicated that the inflammatory response to bacteria is prolonged in db/db mice due to unresolved proinflammatory cytokine expression. Studies conducted with macrophage cell lines (44, 45) and primary macrophages (46, 47) indicate that the diabetic milieu heightens macrophage responsiveness to activating factors like LPS. However, Zyкова et al. (48) has shown that peritoneal macrophages isolated from C57BL/KS-lepr-db/db mice had a diminished cytokine secretion response to LPS + IFN-γ ex vivo. An important difference between our current findings and theirs is that they did not confirm their results in the in vivo setting (i.e., the peritoneum). Their results simply represent ex vivo macrophage treatments. Other contributing factors may also include their use of the C57BL/KS strain which has a more rapid onset of acute disease than the C57BL/6J (used in this study), which develops the characteristics of type 2 diabetes (Table I) without developing acute disease until later in life better modeling human type 2 diabetes. In addition, Zyкова et al. cultured their macrophages ex vivo for up to 3 days, and cells removed from the diabetic environment through ex vivo culture can lose their diabetic phenotype. In our current study, all experiments involving ex vivo macrophages were conducted within 3 h of isolation. Also, unlike Zyкова et al., we did not cotreat macrophages with IFN-γ. Not using IFN-γ pretreatment more accurately represents the immediate macrophage response to pathogens as our quantifying of peritoneal IL-1β levels support (Fig. 3b).

Negative regulation of IL-1β is conducted, in part, via the expression of IL-1ra and IL-1R2. Pretreatment with the receptor antagonist or type 2 receptor is effective in blocking the bioactions of IL-1β (29, 31). In fact, therapeutic administration of IL-1ra is beneficial in the treatment of certain chronic inflammatory diseases, like rheumatoid arthritis (49). Although diabetes induces proinflammation, no one has previously examined the role of IL-1ra and/or IL-1R2 in type 2 diabetes. Fig. 3c indicates that i.p. expression of IL-1ra is markedly lower in db/db mice when compared with db/+ mice, while Fig. 4, a and b, demonstrate that LPS-induced up-regulation of both IL-1ra and IL-1R2 is significantly impaired in the db/db mouse spleen. This result was interesting and supports our Fig. 2 data that show recovery from sickness is delayed in db/db mice. IL-1ra and IL-1R2 are elaborated by macrophages as a negative feedback mechanism after LPS exposure (26, 27). Anti-inflammatory cytokines like IL-4, IL-10, and IFG-1 also induce up-regulation of IL-1ra and IL-1R2. We have previously shown (32) that db/db mice have a blunted signaling response to IL-4 and IGF-1 failing to fully activate PI3K. The mechanism of this inhibition appears to be mammalian target of rapamycin-mediated insulin receptor substrate-2 serine phosphorylation induced by hyperinsulinemia and hyperglycemia (32). Therefore, a potential means by which db/db mice fail to up-regulate IL-1ra and IL-1R2 is macrophage based IL-4 resistance.

IL-1β is the cytokine principally responsible for induction of brain-based immunity in response to peripheral inflammatory stimuli (26). Macrophages exposed to LPS rapidly elaborate IL-1β, and it is this signal that induces sickness behavior (26). As expected from our results in Fig. 2, peritoneal administration of IL-1β induced sickness behavior in db/db mice that was significantly prolonged when compared with that of nondiabetic mice (Fig. 5). The dose of IL-1β chosen was derived from data in Fig. 3b in which we found that 2 h after i.p. LPS administration, peritoneal levels of IL-1β were ~700 and 1400 pg/ml in db/+ and db/db mice, respectively. The IL-1β dose of 1000 pg/ml administered (Fig. 5) generated a peak sickness response slightly less than that seen with LPS in Fig. 2. Interestingly, nondiabetic mice recovered from this dose of IL-1β within 8 h while diabetic mice required nearly 24 h to fully recover. This is in distinct contrast to the LPS experiments in Fig. 2 which showed that both db/+ and db/db mice required over 12 h to recover. The likely reason for this finding is that LPS induces a more complex immune response than IL-1β alone. However, the IL-1β administration data (Fig. 5) demonstrate the importance of failure to up-regulate IL-1ra and IL-1R2 in counterregulation of db/db mouse IL-1β-dependent activation of the central innate immune response.

The way in which abdominal inflammation is initially communicated to the brain is through afferent vagus nerve fibers (26). Subdiaphragmatic vagotomy in rodents prevents both the behavioral depression and activation of the limbic system following i.p. administration of LPS or rIL-1β (35). Induction of central IL-1β expression by peripheral LPS or IL-1β is blocked in vagotomized animals, but when IL-1β is injected centrally, vagotomy has no effect on the activation of the brain based innate immune response (35). Fig. 6, a and b, show that IL-1ra and IL-1R2 fail to up-regulate in the brain of diabetic animals after i.p. LPS administration. Although we (29) and others (31) have shown the importance of IL-1ra and IL-1R2 in controlling neuroimmunity, this study is the first to show that diabetes is associated with failed up-regulation of IL-1ra and/or IL-1R2. In addition, these data are the first to show that in a natural model of disease, loss of IL-1β counterregulation exists. Finally, Fig. 6c demonstrates that when IL-1β is infused into the ventricular system, db/db mice fail to recover from sickness as quickly as nondiabetic animals. These finding supports a direct immune perturbation in the brain and not a failure of vagal communication to the brain in type 2 diabetes.

Disclosures
The authors have no financial conflict of interest.

References