Burkholderia pseudomallei Triggers Altered Inflammatory Profiles in a Whole-Blood Model of Type 2 Diabetes-Melioidosis Comorbidity

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Melioidosis is a potentially fatal disease caused by the bacterium *Burkholderia pseudomallei*. Type 2 diabetes (T2D) is the most common comorbidity associated with melioidosis. *B. pseudomallei* isolates from melioidosis patients with T2D are less virulent in animal models than those from patients with melioidosis and no identifiable risk factors. We developed an *ex vivo* whole-blood assay as a tool for comparison of early inflammatory profiles generated by T2D and nondiabetic (ND) individuals in response to a *B. pseudomallei* strain of low virulence. Peripheral blood from individuals with T2D, with either poorly controlled glycemia (PC-T2D [$n = 6$]) or well-controlled glycemia (WC-T2D [$n = 8$]), and healthy ND ($n = 13$) individuals was stimulated with *B. pseudomallei*. Oxidative burst, myeloperoxidase (MPO) release, expression of pathogen recognition receptors (TLR2, TLR4, and CD14), and activation markers (CD11b and HLA-DR) were measured on polymorphonuclear (PMN) leukocytes and monocytes. Concentrations of plasma inflammatory cytokine (interleukin-6 [IL-6], IL-12p70, tumor necrosis factor alpha [TNF- α], monocyte chemoattractant protein 1 [MCP-1], IL-8, IL-1 β , and IL-10) were also determined. Following stimulation, oxidative burst and MPO levels were significantly elevated in blood from PC-T2D subjects compared to controls. Differences were also observed in expression of Toll-like receptor 2 (TLR2), CD14, and CD11b on phagocytes from T2D and ND individuals. Levels of IL-12p70, MCP-1, and IL-8 were significantly elevated in blood from PC-T2D subjects compared to ND individuals. Notably, differential inflammatory responses of PC-T2D, WC-T2D, and ND individuals to *B. pseudomallei* occur independently of bacterial load and confirm the efficacy of this model of T2D-melioidosis comorbidity as a tool for investigation of dysregulated PMN and monocyte responses to *B. pseudomallei* underlying susceptibility of T2D individuals to melioidosis.

Melioidosis, caused by *Burkholderia pseudomallei* is an increasingly important disease in the tropics (17, 25). Melioidosis is the third most common cause of death from infectious disease in northeast Thailand (37). In northern Australia, melioidosis pneumonia is an increasingly recognized cause of community-acquired pneumonia (19). Rapid progression to septic shock and death are common complications (19). Type 2 diabetes (T2D) is the most significant risk factor associated with susceptibility to infection with *B. pseudomallei*. Up to 42% of patients with melioidosis in Australia (16, 19), 60% of patients in Thailand (37), and 76% of patients in India (48) have preexisting T2D. A considerable proportion (43%) of nondiabetic patients with melioidosis have other underlying risk factors associated with progression to T2D, including increased body mass index (BMI), hypertension, hypertriglyceridemia, and renal problems (Townsville Hospital melioidosis database, R. Norton, unpublished data). There are inconsistencies in reports on the impact of T2D on severity of *B. pseudomallei* infection (19, 30, 55). Given T2D is the most common risk factor for melioidosis and that individuals with preexisting diabetes are more likely to present with acute rather than chronic infection (18, 19), this comorbid condition undoubtedly impacts the ability of the host immune response to control *B. pseudomallei* infection.

The inflammatory nature of insulin resistance and T2D is well documented (52). Defects in cellular immune responses, which tend to be more pronounced in diabetic individuals with poor glycemic control (40, 41, 53, 57), have been described for other infections (33). However, the impact of T2D on immunopathogenesis of *B. pseudomallei* infection remains an underresearched area. We have previously demonstrated variation in virulence levels among clinical isolates of *B. pseudomallei* in animal models

(58). In BALB/c mice, the virulence of *B. pseudomallei* isolates from patients with preexisting T2D is significantly lower than that of isolates recovered from patients with no identifiable risk factors (58), suggesting immunopathological changes associated with diabetes increase susceptibility to otherwise innocuous strains of *B. pseudomallei*.

Recent *in vitro* studies have demonstrated defects in the response of individual leukocyte types from diabetic hosts toward *B. pseudomallei* (11, 45, 65). Compared to healthy controls, mononuclear leukocytes isolated from peripheral blood of diabetic individuals express lower levels of interleukin-17 (IL-17) following exposure to *B. pseudomallei* (45). Recently, we demonstrated that bone marrow-derived dendritic cells (BMDC) and peritoneal elicited macrophages (PEM) isolated from streptozotocin-induced diabetic mice are impaired in their ability to internalize and kill *B. pseudomallei* *in vitro* (65). In addition, polymorphonuclear leukocytes (PMN) from patients with melioidosis and comorbid T2D have been shown to have impaired phagocytosis and migration in response to IL-8 and a reduced ability to delay apoptosis following exposure to *B. pseudomallei* (11). Defects in PMN function in T2D, excess alcohol consumption, and renal disease are well de-

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scribed and were the basis for trial therapy with granulocyte colony-stimulating factor (G-CSF) in melioidosis (12, 14, 31, 46, 67). However, a disadvantage of functional assays using a single leukocyte type in isolation is the inability to consider the modulating effects of other cell types and plasma constituents on inflammatory responses important in controlling *B. pseudomallei* infection.

Therefore, in the present study, we developed an *ex vivo* whole-blood assay and characterized its efficacy as a tool for assessing early inflammatory responses toward *B. pseudomallei* infection in susceptible hosts. We compared bacterial loads and inflammatory profiles in peripheral blood of individuals with T2D and in healthy individuals without T2D following exposure to a *B. pseudomallei* strain with low virulence in an animal model of melioidosis (5). In addition, we sought to determine the influence of glycemic control on inflammatory responses to *B. pseudomallei*, by comparing profiles in blood from individuals with either poorly controlled (PC) or well-controlled (WC) T2D. Comparable bacterial numbers were demonstrated for all groups throughout the experimental period. Importantly, however, we demonstrate contrasting expression of pathogen recognition receptors (PRRs; e.g., Toll-like receptor 2 [TLR2], TLR4, and CD14) and activation markers (CD11b, HLA-DR) on PMN and monocytes from T2D individuals, particularly those with poorly controlled glycemia, and healthy controls in response to stimulation with *B. pseudomallei*. Similarly, exposure to *B. pseudomallei* led to differences in the secretion of inflammatory cytokines between controls and individuals with T2D. Parallels between the findings of the present study with those described from patients (62) and animal models (6, 32, 59) support a role for our whole-blood model of diabetes-melioidosis comorbidity as a valuable tool in investigating impaired early host cell interactions that underlie susceptibility to melioidosis.

MATERIALS AND METHODS

Participants. A total of 14 individuals with diabetes were recruited through the outpatient Endocrinology Clinic of the Townsville Hospital, Queensland, Australia. T2D was diagnosed according to the World Health Organization criteria (66). Participants with T2D consisted of 6 males and 8 females, aged 31 to 78 years (mean, 54.4 years, standard deviation [SD], 12.6 years). The mean duration from the first diagnosis of diabetes was 11.8 years (SD, 8.9; range, 6 months to 33 years). Participants with T2D were administered either sulfonylurea (8 patients), α -glucosidase (2 patients), or biguanide (9 patients) or were administered no medication, being under dietary control only (3). Individuals with diabetes were subdivided based on their history of glycemic control, as indicated by the percentage of glycosylated hemoglobin (HbA1c) on monthly clinic visits over a period of at least 6 months: poorly controlled (PC-T2D; HbA1c > 8.5%; $n = 6$) or well controlled (WC-T2D; HbA1c = 5.5 to 7.5%; $n = 8$). Healthy, nondiabetic (ND) controls were age and gender matched (mean age, 56 years; SD, 11.7 years; range, 29 to 76 years). All participants—those with diabetes or healthy controls—had no history of melioidosis and were seronegative for antibodies against *B. pseudomallei* according to an indirect hemagglutination assay (3).

Venous blood (40 ml) was obtained from participants in sodium heparin Vacutainer tubes (Becton Dickinson). Experiments were performed over a series of runs, where each run consisted of at least one diabetic individual age and gender matched to a healthy control individual. This study was approved by the human ethics review committees of the Townsville Hospital (71/04) and James Cook University (H3483). All participants gave informed consent.

Biochemical and hematological measurements. Blood cell counts, including numbers and proportions of leukocytes, erythrocyte sedimentation rate (ESR), HbA1c, and C-reactive protein (CRP) were determined in unstimulated peripheral blood by the routine diagnostic laboratory at the Townsville Hospital.

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Whole-blood stimulation assay. A previously characterized (5) *B. pseudomallei* clinical strain of low virulence (NCTC13179) was used for *in vitro* stimulation in whole-blood assays. The isolate was grown to the logarithmic phase, washed twice, and resuspended in phosphate-buffered saline (PBS; pH 7.2) to 4×10^8 CFU/ml, as described previously (5). *B. pseudomallei* was added to whole blood for the assays described below at a multiplicity of infection (MOI) of 1:5 (1 leukocyte to 5 bacteria). Unstimulated, control samples received an equivalent volume of PBS only. Stimulated and unstimulated whole blood was incubated at 37°C in 5% CO₂ with gentle mixing for up to 3.5 h.

***B. pseudomallei* persistence.** Persistence of *B. pseudomallei* throughout the culture period was monitored in stimulated blood samples by measuring total and intracellular bacterial loads at 1 and 4 h of incubation. The total *B. pseudomallei* concentration (intracellular and extracellular) was determined by culturing serial dilutions of whole blood onto Ashdown agar at the time points indicated. Intracellular bacterial loads were determined following lysis of erythrocytes in erythrocyte lysis buffer (Qiagen) and washing of the remaining leukocytes. Leukocytes were resuspended to the original volume, and serial dilutions were plated onto Ashdown agar. Colonies were enumerated after 48 h of culture, and the *B. pseudomallei* load was determined. Assays were performed in duplicate at each time point for all samples. Data for total bacterial load are expressed as log₁₀ CFU/ml of blood. Intracellular bacterial loads are expressed as log₁₀ CFU/10⁶ leukocytes.

Flow cytometric analysis of PMN and monocyte cell surface activation markers. Using flow cytometry, we compared the levels of activation of blood phagocytes from PC-T2D, WC-T2D, and ND individuals following exposure to *B. pseudomallei*. The PRRs TLR2, TLR4, and CD14 have previously been demonstrated to play a role in the pathogenesis of melioidosis (61, 63, 64). We therefore assessed their expression on PMN and monocytes prior to and following stimulation with *B. pseudomallei*. In addition, blood phagocyte activation was compared for PC-T2D, WC-T2D, and ND individuals by measuring changes in the expression of cell surface activation markers on PMN (CD11b) and monocytes (CD11b and HLA-DR) following stimulation with *B. pseudomallei*. Monoclonal antibodies were purchased from BD Biosciences (CD45, CD3, CD16, CD14, HLA-DR, and isotype controls) and Jomar Biosciences (CD11b and isotype control) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin-chlorophyll protein (PerCP). Toll-like receptor 2 (TLR2)-Alexa Fluor 488 and TLR4-biotinylated (detected with streptavidin-conjugated PE) antibodies were also purchased from BD Biosciences. All monoclonal antibodies, except TLR4, were employed in direct immunofluorescence tests using whole blood according to the manufacturers' instructions. Briefly, appropriate monoclonal antibodies were added to 100 μ l of whole blood and incubated on ice for 30 min. Erythrocyte lysing solution (1 $\times$ FACSlyse; BD Biosciences) was added for 10 min at room temperature. Samples were washed twice then resuspended in 2% paraformaldehyde. Immunofluorescence-positive cells were determined by flow cytometry (BD FACScan, BD Biosciences). PMN activation was assessed by CD11b, TLR2, and TLR4 expression after gating on CD16⁺ granulocytes. Monocyte activation was assessed by CD11b, CD14, HLA-DR, TLR2, and TLR4 expression after gating on CD45⁺ CD3[−] mononuclear leukocytes. Sample analysis was performed with CellQuestPro software (BD Biosciences). Data are expressed as mean fluorescence intensity in unstimulated (unstim) and *B. pseudomallei*-stimulated (stim) samples.

MPO activity. The release of myeloperoxidase (MPO) from activated PMN was measured in plasma from stimulated and unstimulated samples at 1 and 3.5 h of culture using a sandwich enzyme-linked immunosorbent assay (ELISA) for human MPO according to the manufacturer's protocol (Hycult Biotech). Samples were analyzed in duplicate in the same run.

Oxidative burst activity. Intracellular oxidative burst activity of PMN and monocytes was determined by flow cytometry using the fluorogenic substrate dihydrorhodamine 123 (DHR; Invitrogen). One hundred microliters of whole blood was mixed with *B. pseudomallei* at an MOI of 1:5 (or PBS only for unstimulated control samples) for 30 min. DHR (100 μ M) was added to the samples at 37°C, and incubation was continued for a further 10 min in order to allow nonfluorescent DHR to convert to fluorescent rhodamine 123 upon the production of reactive oxygen species. Lysing solution (1 $\times$ FACSlyse) was added for 10 min at room temperature. After washing, CD16-PE was added to samples and incubated for 20 min on ice. Samples were washed twice and resuspended in 2% paraformaldehyde for flow cytometric analysis. Granulocyte populations were gated on forward versus side scatter plots, followed by gating on CD16⁺ granulocytes. Monocytes were gated on forward versus side scatter plots. Oxidative burst was monitored by determining the proportion of cells positive for rhodamine 123 and the relative fluorescence intensities of the gated PMN or monocytes.

Inflammatory cytokine analysis. At 1 and 3.5 h of incubation, 1-ml aliquots of *B. pseudomallei*-stimulated and unstimulated blood were centrifuged (500 $\times$ g, 5 min). Plasma was removed and stored at -70°C until further analysis. Inflammatory cytokines were measured with the BD Biosciences CBA kit: IL-8, IL-1 β , IL-6, IL-10, tumor necrosis factor alpha (TNF- α), and IL-12p70 (human inflammatory cytokines) using a BD FACScan flow cytometer (BD Biosciences) and a BD Biosciences CBA MCP-1 Flex kit with a BD FACSCalibur flow cytometer (BD Biosciences), according to the manufacturers' instructions. Assays were performed in duplicate at each time point for all samples. Cytokine concentration (pg/ml) was determined with calibration curves separately established using the CBA analysis software (BD Biosciences).

Statistical analysis. Differences in inflammatory responses of unstimulated and *B. pseudomallei*-stimulated samples were examined using Wilcoxon's signed rank test. Differences in inflammatory responses between the PC-T2D, WC-T2D, and ND groups were calculated using Kruskal-Wallis test. Simple linear correlations between HbA1c, as a marker of glycemic control, and immunological measures at 3.5 h of culture were determined by Spearman's rank correlation coefficient. A *P* value of <0.05 was considered significant. All statistical analyses were conducted using Graphpad Prism v5 software. Results are expressed as the median $\pm$ interquartile range (IQR) unless otherwise specified.

RESULTS

Clinical, biochemical, and hematological characteristics. Compared to ND individuals, ESR, CRP, and leukocyte numbers were elevated in unstimulated blood from PC-T2D individuals (Table 1). Increased leukocyte levels corresponded to neutrophilia in PC-T2D individuals.

***B. pseudomallei* persistence.** Growth kinetics of *B. pseudomallei* in whole blood were comparable for PC-T2D, WC-T2D, and ND individuals throughout the experimental period (see Fig. S1 in the supplemental material). Following stimulation, there were no significant differences in total or intracellular bacterial loads between the three groups at 1 or 4 h of culture.

PMN activation. Following *B. pseudomallei* stimulation, the percentage of PMN undergoing oxidative burst (Fig. 1A) increased significantly for all groups (42-fold for PC-T2D, 25-fold for WC-T2D, and 36-fold for ND individuals). While there was a trend for greater levels of oxidative burst activity for PMN from PC-T2D compared to ND and WC-T2D individuals, this did not reach statistical significance. Levels of MPO, an enzyme that is liberated from activated PMN, were similarly increased in *B. pseudomallei*-stimulated blood samples (Fig. 1B) and were significantly higher in blood from PC-T2D individuals compared to ND

TABLE 1 Clinical, biochemical, and hematological characteristics of the individuals in this study^a

Characteristic	Result for group:		
	PC-T2D	WC-T2D	ND
<i>n</i>	6	8	13
No. (males/females)	2/4	4/4	6/7
BMI (kg/m ²) (mean $\pm$ SD)	40.8 $\pm$ 4.5*	34.0 $\pm$ 5.9	—
Family history of diabetes (%)	100	75	—
TC (mmol/liter)	4.9 $\pm$ 0.6	4.5 $\pm$ 1.3	—
TG (mmol/liter)	2.5 $\pm$ 1.4	1.8 $\pm$ 0.9	—
HbA1c (%)	10.1 $\pm$ 1.2**	6.1 $\pm$ 0.9	5.1 $\pm$ 0.4
ESR (mm/hour)	32.8 $\pm$ 18.4**	42.1 $\pm$ 24†	10.7 $\pm$ 7.6
CRP (mg/liter)	8.7 $\pm$ 7.3**	7.0 $\pm$ 5.2	2.0 $\pm$ 0.9
RBCC (10 ¹² /liter)	4.9 $\pm$ 0.6	4.6 $\pm$ 0.3	4.8 $\pm$ 0.5
Hemoglobin (g/liter)	136.7 $\pm$ 14.9	132.5 $\pm$ 13.4	139.4 $\pm$ 16.0
Hematocrit	0.42 $\pm$ 0.03	0.4 $\pm$ 0.03	0.42 $\pm$ 0.4
MCV (femtoliter)	86.3 $\pm$ 7.6	88.3 $\pm$ 4.8	88.4 $\pm$ 7.3
Platelets (10 ⁹ /liter)	270.8 $\pm$ 91.7	241.8 $\pm$ 117.7	236.5 $\pm$ 47.7
WBCC (10 ⁹ /liter)	8.05 $\pm$ 2.5**	6.4 $\pm$ 1.4	5.6 $\pm$ 1.1
Lymphocytes (10 ⁹ /liter)	2.25 $\pm$ 0.39	1.75 $\pm$ 0.41	1.84 $\pm$ 0.48
Monocytes (10 ⁹ /liter)	0.59 $\pm$ 0.21	0.54 $\pm$ 0.14	0.45 $\pm$ 0.11
Neutrophils (10 ⁹ /liter)	4.86 $\pm$ 2.2**	3.84 $\pm$ 1.13	3.04 $\pm$ 0.72

^a WC, well-controlled glycemia; PC, poorly controlled glycemia; T2D, type 2 diabetes; ND, nondiabetic control individuals; BMI, body mass index; HbA1c, glycated hemoglobin A1c; TC, total cholesterol; TG, triglycerides; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RBCC, red blood cell count; MCV, mean corpuscular volume; WBCC, white blood cell count. —, not determined. Nominal variables are presented as numbers; continuous variables are presented as the mean $\pm$ standard deviation. Continuous variables were compared between groups by using Mann-Whitney *U* test. *, PC-T2D compared to WC-T2D, *P* < 0.05; **, PC-T2D compared to ND, *P* < 0.05; †, WC-T2D compared to ND, *P* < 0.05.

individuals (*P* = 0.05; 6.5-fold versus 4.7-fold increase for PC-T2D and ND individuals, respectively).

We also measured surface expression of the integrin, CD11b, as a marker of PMN activation. CD11b levels increased on PMN from all groups following exposure to *B. pseudomallei* (Fig. 1C). There was a trend toward lower CD11b expression on PMN from PC-T2D individuals following stimulation, compared to WC-T2D (*P* = 0.08) and ND (*P* = 0.094) individuals.

Levels of TLR2 expression were comparable on PMN from PC-T2D, WC-T2D, and ND individuals at baseline (Fig. 1D). After exposure to *B. pseudomallei*, TLR2 expression increased significantly for all groups, with the highest levels observed on PMN from ND individuals (Fig. 1D). Minimal TLR4 expression was detected on PMN at baseline among the three groups, and these levels remained relatively unchanged following exposure to *B. pseudomallei* (data not shown).

Monocyte activation. Following stimulation, the percentage of DHR⁺ monocytes (Fig. 2A) and the intensity of the oxidative burst (data not shown) increased significantly for all groups, with the highest fold change observed for WC-T2D individuals (12.8-fold) compared to PC-T2D (4.3-fold) and ND (8.7-fold) individuals. There was a trend toward higher levels of oxidative burst activity in monocytes from PC-T2D individuals compared to monocytes from ND individuals (*P* = 0.067) following stimulation. Monocyte oxidative responses to *B. pseudomallei* reflected the same trends observed in PMN, whereby both the number of cells undergoing oxidative burst activity and the intensity of the oxidative burst response were increased

FIG 1 Biomarker levels on PMN from PC-T2D, WC-T2D, and ND individuals prior to (unstim) and following exposure to (stim) to *B. pseudomallei* (MOI of 1:5 [1 leukocyte to 5 bacteria]). (A and B) The percentage of PMN undergoing oxidative burst (A) and the plasma concentration of MPO released from PMN (B) were determined. In addition, expression of CD11b (C) and TLR2 (D) was determined as measures of PMN activation in response to *B. pseudomallei* stimulation. MFI, mean fluorescent intensity. Results are expressed as the median $\pm$ IQR. *, $P < 0.05$.

in individuals with T2D, particularly those with poorly controlled glycemia.

Compared to PMN, there was only a slight increase in CD11b expression on monocytes following exposure to *B. pseudomallei* (Fig. 2B), with significant increases observed only on monocytes from WC-T2D ($P = 0.023$) and ND ($P = 0.048$) individuals. In contrast, CD14 expression decreased significantly on monocytes from PC-T2D ($P = 0.031$), WC-T2D ($P = 0.039$), and ND ($P = 0.013$) individuals following stimulation with *B. pseudomallei* (Fig. 2C). While minimal changes were observed in HLA-DR expression on monocytes from PC-T2D and ND individuals after exposure to *B. pseudomallei*, a significant increase in HLA-DR levels occurred on monocytes from WC-T2D individuals following stimulation ($P = 0.003$) (Fig. 2D).

Compared to ND individuals, there was a trend for increased TLR2 expression on resting, unstimulated monocytes from both PC-T2D and WC-T2D individuals (Fig. 2E). Following exposure to *B. pseudomallei*, expression of TLR2 decreased on monocytes from PC-T2D (1.1-fold) and WC-T2D (1.1-fold) individuals and remained relatively unchanged on monocytes from ND individuals, although these changes were not significant. No significant differences were observed in baseline TLR4 expression on monocytes from T2D and ND individuals (Fig. 2F). However, com-

pared to monocytes from ND individuals, TLR4 expression was increased on monocytes from PC-T2D individuals ($P = 0.039$) in response to *B. pseudomallei* exposure.

Inflammatory cytokines. There were no statistically significant differences in baseline plasma concentrations of IL-1 β , IL-8, IL-10, or IL-12p70 between PC-T2D, WC-T2D, and ND individuals. However, compared to WC-T2D individuals, IL-6 ($P = 0.031$) and TNF- α ($P = 0.001$) concentrations were elevated in baseline plasma from PC-T2D individuals (Fig. 3A and C). There was also a trend toward increased MCP-1 levels in baseline plasma from PC-T2D individuals compared to plasma from ND individuals ($P = 0.083$) (Fig. 3D).

Following stimulation of whole blood with *B. pseudomallei* for 1 h, plasma concentrations of IL-1 β , IL-8, IL-10, IL-12p70, IL-6, and TNF- α increased significantly for all groups, although levels were similar between PC-T2D, WC-T2D, and ND individuals (see Fig. S2 in the supplemental material). MCP-1 levels remained unchanged at 1 h poststimulation (data not shown). After 3.5 h of exposure to *B. pseudomallei*, significant increases in levels of IL-6 (Fig. 3A) (237-fold, 218-fold, and 181-fold for PC-T2D, WC-T2D, and ND individuals, respectively), TNF- α (Fig. 3C) (141-fold, 447-fold, and 86-fold for PC-T2D, WC-T2D, and ND individuals, respectively), and IL-1 β (92-fold, 116-fold, and 78-fold

FIG 2 Biomarker levels on monocytes from PC-T2D, WC-T2D, and ND individuals prior to (unstim) and following exposure to (stim) *B. pseudomallei* (MOI of 1:5 [1 leukocyte to 5 bacteria]). (A) The percentage of monocytes undergoing oxidative burst was determined. Monocyte activation was also compared among groups by determining changes in expression of CD11b (B), CD14 (C), HLA-DR (D), TLR2 (E), and TLR4 (F) prior to and following exposure to *B. pseudomallei*. MFI, mean fluorescent intensity. Results are expressed as the median $\pm$ IQR. *, $P < 0.05$.

for PC-T2D, WC-T2D, and ND individuals, respectively) were observed for all groups. IL-12p70 concentrations were significantly higher in plasma from PC-T2D individuals compared to WC-T2D ($P = 0.005$) and ND ($P = 0.039$) individuals following stimulation for 3.5 h. Similarly, exposure to *B. pseudomallei* led to elevated levels of IL-8 in plasma from PC-T2D compared to ND individuals ($P = 0.032$; 182-fold versus 43-fold, respectively) at 3.5 h poststimulation. IL-10 concentrations also in-

creased in *B. pseudomallei*-stimulated blood, although differences only reached significance for PC-T2D (1.5-fold) and WC-T2D (1.6-fold) individuals (Fig. 3F).

Effect of HbA1c on inflammatory profiles. We analyzed the effect of glycemic control, indicated by HbA1c levels, on inflammatory biomarkers following *B. pseudomallei* exposure (Table 2). TLR2 expression on PMN correlated negatively with HbA1c levels ($P = 0.041$). In contrast, following exposure to *B. pseudomallei*, the per-

FIG 3 Inflammatory cytokine production in whole blood from PC-T2D, WC-T2D, and ND individuals prior to (unstim) and following 3.5 h of exposure (stim) to *B. pseudomallei* (MOI of 1:5 [1 leukocyte to 5 bacteria]). Plasma concentrations of IL-6 (A), IL-12p70 (B), TNF- α (C), MCP-1 (D), IL-8 (E), and IL-10 (F) were measured in duplicate. Data represent the median cytokine concentration (pg/ml) in unstimulated and stimulated blood within each participant group (ND, WC-T2D, and PC-T2D) $\pm$ IQR. *, $P < 0.05$.

centage of monocytes undergoing respiratory burst ($P = 0.027$) and the concentrations of MCP-1 ($P = 0.002$) and IL-8 ($P = 0.005$) correlated positively with HbA1c levels. No significant correlation was observed between age, gender, or time since diagnosis of T2D and inflammatory markers following exposure to *B. pseudomallei* (data not shown).

DISCUSSION

It is widely recognized that T2D is associated with increased rates of infection and infection-related mortalities (33, 50). However,

there is a paucity of literature concerning diabetes-induced immune alterations and their effect on subsequent host-pathogen interactions. Despite being the most significant risk factor associated with susceptibility to melioidosis, the impact of preexisting diabetes on the immunopathogenesis of *B. pseudomallei* infection is unclear. Bacteremia and sepsis are common clinical findings of melioidosis (18). We had previously demonstrated that compared to individuals with no identifiable risk factors, individuals with T2D are more susceptible to infection with less virulent *B. pseudomallei* isolates (58). Therefore, using a whole-blood stimulation

TABLE 2 Association of percentage of HbA1c as an indicator of glycemic control with inflammatory biomarkers following exposure to *B. pseudomallei*

Inflammatory biomarker type	95% CI ^a	Spearman's <i>r</i>	<i>P</i> value ^b
PMN			
Oxidative burst (DHR)	−0.283–0.462	0.104	0.592
TLR2	−0.663–0.007	−0.382	0.041*
CD11b	−0.578–0.132	−0.257	0.177
Monocytes			
Oxidative burst (DHR)	0.040–0.681	0.410	0.027*
TLR2	−0.332–0.419	0.050	0.795
TLR4	−0.109–0.593	0.279	0.143
CD11b	−0.553–0.167	−0.223	0.245
CD14	−0.454–0.307	−0.086	0.664
HLA-DR	−0.358–0.395	0.022	0.911
Cytokines			
IL-6	−0.133–0.598	0.271	0.171
IL-12p70	−0.180–0.556	0.226	0.256
TNF- α	−0.071–0.637	0.328	0.095
MCP-1	0.235–0.781	0.567	0.002*
IL-8	0.168–0.759	0.524	0.005*
IL-10	−0.221–0.537	0.186	0.354
IL-1 β	−0.423–0.356	−0.040	0.844

^a CI, confidence intervals.^b *, significant at *P* < 0.05.

assay, the present study is the first to demonstrate differences in inflammatory profiles of leukocytes from whole blood of diabetic and nondiabetic individuals in response to a *B. pseudomallei* strain of low virulence, despite comparable bacterial loads. While strong inflammatory responses were induced in blood from both diabetic subjects and healthy controls, exposure to *B. pseudomallei* led to increased levels of IL-12p70, MCP-1, IL-8, and IL-10 in blood from individuals with T2D, particularly those with poorly controlled glycemia. Interestingly, differences were also observed in PRR and cell surface activation marker expression on phagocytes from diabetic and nondiabetic individuals. It is noteworthy that the contrasting inflammatory responses were not a reflection of *B. pseudomallei* numbers, since total and intracellular bacterial loads were comparable for diabetics and nondiabetic individuals throughout the experimental period. Given the importance of PRR signaling in determining the subsequent activation of diverse immune response pathways, the contrasting expression identified on phagocytes in the present study may underpin the susceptibility of T2D individuals to melioidosis.

Chronic low-grade inflammation and oxidative stress are well documented in T2D and play an important role in the development of many of the metabolic complications associated with insulin resistance (27, 44). In addition to many metabolic abnormalities, a variety of immunological abnormalities continue to be identified that conceivably contribute to the increased susceptibility of T2D individuals to infections such as melioidosis (35, 44). Prolonged hyperglycemia, oxidative stress, and production of advanced glycated end products contribute to local production of inflammation and tissue damage and delayed wound repair in diabetics (68). In the present study, unstimulated blood from individuals with T2D, particularly those with poorly controlled glycemia, had increased erythrocyte sedimentation rates, C-reactive

protein, TNF- α , and IL-6 levels, all of which are consistent with an underlying inflammatory state which has been shown to occur in association with T2D (35, 43, 44).

Recruitment, migration, and activation of leukocyte subsets are crucial events during an immune response that are regulated by chemokines and inflammatory cytokines. However, while these mediators are indispensable for the effective clearance of a pathogen, an excessive inflammatory response is potentially destructive and may contribute to disease progression (60). The proinflammatory cytokine profiles observed in the present study are consistent with those described in melioidosis sepsis (29, 62), and both IL-6 and IL-10 have been shown to be independent predictors of mortality (51). Interestingly, levels of MCP-1 and IL-8 correlated with glycemic control, with the highest concentrations measured in plasma from PC-T2D individuals (high percentage of HbA1c). In contrast to WC-T2D and ND individuals, IL-12p70 was also elevated in plasma from PC-T2D individuals following stimulation with *B. pseudomallei*. It is noteworthy that the anti-inflammatory cytokine IL-10 increased significantly in blood from T2D individuals. To our knowledge, there have been no previous published comparisons of inflammatory cytokine production between diabetic and nondiabetic melioidosis patients. Wiersinga et al. (62) did, however, report the increased expression of mRNA for genes encoding IL-1 β , IL-6, TNF- α , gamma interferon (IFN- γ), and IL-10 in peripheral blood leukocytes from patients with melioidosis sepsis. In contrast to the present study, MCP-1 and IL-8 levels were lower in monocytes of patients with melioidosis sepsis compared to those in controls (62). However, comparisons of cytokine responses between the *ex vivo* model used in the present study and that of Wiersinga et al. (62) are not possible given that peripheral blood from the melioidosis sepsis study was collected from melioidosis patients after the commencement of antimicrobial therapy. Nevertheless, despite differences in the focus of the study by Wiersinga et al. (62) and our present study, both have demonstrated the induction of both proinflammatory and anti-inflammatory cytokines in leukocytes from patients with melioidosis sepsis and those from susceptible T2D individuals, respectively, after stimulation with *B. pseudomallei*.

We have previously demonstrated differential inflammatory cytokine responses in our animal models of melioidosis, in which BALB/c mice are highly susceptible to *B. pseudomallei* infection and C57BL/6 mice are relatively resistant (6, 59). Differences have been described in the kinetics and magnitude of keratinocyte chemoattractant (KC), a homologue of IL-8 in humans, MCP-1, IL-12, and IL-10 mRNA induction between the two mouse strains (6, 59). Compared to C57BL/6 mice, susceptible BALB/c mice had elevated inflammatory chemokine and cytokine responses in liver and spleen within 48 h of infection with *B. pseudomallei*, corresponding to a more extensive inflammatory cell infiltrate that is rich in PMN (6, 59). Paradoxically, the increased inflammatory response to *B. pseudomallei* in BALB/c mice is associated with increased bacterial dissemination and mortality (6, 59). This suggests that the exaggerated inflammatory cytokine production and PMN infiltration to sites of *B. pseudomallei* infection, apparent by day 2 of infection, may be a final, desperate response of the susceptible host following failed earlier attempts at controlling pathogen replication. Recently, we characterized for the first time an animal model of T2D melioidosis comorbidity (32) and described a disease pattern in diabetic mice that is similar to that observed in the susceptible BALB/c mice. Compared to nondia-

betic mice, in diabetic mice, hyperproduction of proinflammatory cytokines, including TNF- α , IL-1 β , and IL-6, and extensive PMN infiltration and tissue damage at sites of infection preceded mortality in the first few days following *B. pseudomallei* infection (32). Data from the present study demonstrate that the levels of control of *B. pseudomallei* persistence within the first few hours of exposure are similar for PC-T2D, WC-T2D, and ND individuals. Importantly, despite comparable bacterial loads, the inflammatory pathways triggered in leukocytes in response to this bacterium differ significantly for T2D and ND individuals.

In the present study, there was evidence of lower proinflammatory cytokines in blood from WC-T2D compared to PC-T2D individuals. Given the inflammatory nature of diabetes, there has been increasing interest in the identification of compounds with antioxidative and anti-inflammatory properties in the treatment of infection in individuals with diabetes (10). This is supported by recent evidence that for patients with melioidosis sepsis, those with preexisting T2D that were taking glyburide, a sulfonylurea with anti-inflammatory properties, were more likely to survive than nondiabetics or T2D individuals receiving other antidiabetic treatments prior to diagnosis with melioidosis (34). T2D individuals recruited to the present study were receiving one or more of a variety of antidiabetic medication classes, including sulfonylureas, α -glucosidases, and biguanides. Unfortunately, this meant that group sizes were inadequate for analysis of the potential effects of antidiabetic medication on inflammatory profiles generated in response to *B. pseudomallei* exposure. Nevertheless, the findings in the present study have clearly demonstrated that glycemic control does influence the host inflammatory response to *B. pseudomallei*. The hyperinflammatory responses observed between 24 and 48 h in susceptible BALB/c mice (6, 59), in diabetic mice (32), and in patients with melioidosis sepsis (62) reflect the inflammatory profiles observed in the whole-blood assay used in the present study. Based on our findings, we propose that very early interactions between *B. pseudomallei* and PMN and monocytes are altered in the diabetic host, such that a compensatory dysregulated hyperinflammatory response is triggered independently of bacterial load, resulting in increased local tissue damage and bacterial dissemination.

Both PMN and monocytes are vulnerable to functional disturbances due to hyperglycemia-induced stress (33, 35, 68). Oxidative stress is well described in diabetes, contributing to endothelial dysfunction and vascular inflammation (20). Trends for increased oxidative burst activity observed in PMN and monocytes from PC-T2D individuals both in unstimulated and stimulated blood compared to healthy controls are consistent with the findings of others (4, 23). MPO, a principle enzyme released by activated PMN, was significantly elevated in plasma from PC-T2D individuals compared to ND individuals following exposure to *B. pseudomallei*.

PMN migration from the bloodstream into sites of infection within tissues occurs rapidly through a process that is tightly regulated. Activated PMN display an upregulated expression of CD11b and other integrins and chemokine receptors, which facilitate their transmigration into sites of infection within tissue (15). Reduced PMN function has been demonstrated in patients with melioidosis and comorbid T2D (11). Based on the association of PMN functional defects with the most common underlying risk factors for melioidosis, G-CSF, which stimulates the production and function of PMN, was used in a trial as an adjunctive therapy

for the treatment of septic shock from melioidosis (12, 14, 31, 46, 67). Although G-CSF had no effect on mortality for patients with severe melioidosis sepsis (12), its efficacy in treatment of patients with less severe forms of melioidosis has not been investigated. In the present study, we examined CD11b expression, which mediates inflammation by regulating leukocyte adhesion and migration, as additional measures of PMN and monocyte activation in response to *B. pseudomallei* exposure. CD11b was upregulated on PMN from all groups after stimulation with *B. pseudomallei*, similar to responses described in sepsis patients (38). However, there was a trend toward reduced levels of CD11b on PMN from PC-T2D individuals at baseline and following stimulation, supporting decreased migratory capacity previously described for PMN from T2D individuals (11, 28). In contrast, CD11b expression was comparable for monocytes from T2D and ND individuals prior to and following stimulation with *B. pseudomallei*. In addition to CD11b, we measured changes in expression of HLA-DR as cell surface markers of monocyte activation. HLA-DR, or the major histocompatibility complex (MHC) class II surface receptor, has a central role in antigen-specific interactions between monocytes and lymphocytes. Similar to CD11b, HLA-DR levels remained relatively unchanged in PC-T2D and ND individuals. However, it remains to be determined whether other integrins or chemokine receptors involved in monocyte activation are altered in diabetic individuals following exposure to *B. pseudomallei*.

PRRs play an important role in the activation and regulation of the innate immune response. TLRs are transmembrane proteins that mediate host cell signaling in response to a range of extracellular and endosomal pathogen-associated molecules. TLR4 permits signaling in response to lipopolysaccharide (LPS) and non-infective inflammatory stimuli, such as heat shock protein 60 (HSP60) (reviewed in reference 42). TLR2, which can heterodimerize with other TLRs, responds to a more diverse group of ligands, including lipoproteins, peptidoglycans, and atypical LPS (reviewed in reference 41). *B. pseudomallei* has been shown to interact with CD14, TLR2, and TLR4 (61, 63, 64), and our group recently identified differences in TLR2 and TLR4 expression on monocytes following stimulation with *B. pseudomallei* isolates of low and high virulence (M. Feterl and N. Ketheesan, unpublished data). TLRs have also been implicated in the pathogenesis of insulin resistance and its complications, with observations that TLR2 and TLR4 expression is increased on monocytes from diabetic individuals (21, 22). Endogenous ligands, such as fatty acids, heat shock proteins, hyaluronan, and high-mobility group B1 protein induce the production of proinflammatory cytokines through the stimulation of TLR2 and TLR4 in individuals with diabetes (21). Given the importance of TLR2, TLR4, and CD14 in melioidosis, we therefore investigated changes in the expression of these pathogen recognition receptors on phagocytes from T2D and ND individuals in response to *B. pseudomallei* stimulation. CD14, one of the first-described PRRs, exists in a membrane-bound form on monocytes, where it serves as a ligand-binding component of the LPS-receptor complex (7). Membrane-bound CD14 can also be shed into a soluble form upon monocyte activation (7). CD14 expression decreased on monocytes exposed to *B. pseudomallei*, with greater decreases observed for monocytes from T2D individuals compared to ND individuals. Changes in CD14 expression may reflect internalization of membrane-bound CD14 (39) or increased shedding of surface CD14 (48). Decreased membrane CD14 on monocytes, described as monocyte hypore-

sponsiveness, has been shown to correlate with sepsis severity (9, 49). Our findings of decreased surface expression of CD14 on monocytes from T2D individuals suggest alterations in monocyte functional capacity following exposure to *B. pseudomallei* may contribute to more severe disease progression in T2D individuals. This is consistent with the clinical observation that individuals with diabetes are more likely to present with melioidosis sepsis than individuals with no identifiable risk factors (19).

TLR4 expression on PMN was low, as previously reported (36). In contrast, TLR2 expression increased on PMN from both T2D and ND individuals after stimulation, with levels tending to be greatest on PMN from healthy controls. On monocytes, TLR4 levels increased marginally following *B. pseudomallei* exposure, with no significant differences observed between groups. While TLR2 expression remained relatively unchanged on monocytes from ND individuals in response to *B. pseudomallei* stimulation, there was a trend for decreased TLR2 expression on monocytes from both PC-T2D and WC-T2D individuals. The pattern of decreased TLR2 and CD14 levels on monocytes from diabetic individuals in the present study is consistent with the profile described for patients with severe sepsis, although no comparisons have been made between diabetic and nondiabetic patients (9, 49, 62). In addition, peripheral blood samples from the sepsis studies were collected from patients following commencement of antimicrobial therapy after hospital admission, making it difficult to draw parallels to the early changes in TLR expression measured in our whole-blood stimulation assay.

The MyD88-dependent pathway is activated by TLR2 and TLR4, resulting in the production of proinflammatory cytokines such as TNF- α , IL-6, and IL-8 (reviewed in reference 42). TLR4 also activates the Toll/IL-1 receptor (TIR) domain-containing adaptor-inducing IFN- β (TRIF)-dependent pathway leading to increased production of IL-12, IL-10, and IFN- β (reviewed in reference 42). Compared to ND individuals, plasma from PC-T2D individuals had elevated levels of IL-12 and IL-10, in addition to higher concentrations of TNF- α , IL-6, and IL-8, suggesting that both MyD88- and TRIF-dependent pathways had been activated. This is supported by the observation of slight increases in TLR4 expression and decreased CD14 activation on monocytes from PC-T2D individuals after exposure to *B. pseudomallei*. Given the contrasting TLR and CD14 activation and inflammatory cytokine response generated in leukocytes from PC-T2D and ND individuals in the present study, it is likely that future kinetic studies focusing on PRR activation in response to *B. pseudomallei* will demonstrate differences within these groups that may be important in directing the generation of subsequent inflammatory responses.

TLR activation not only influences PMN survival, but also has a regulatory effect on chemokine receptor expression on leukocytes, thereby influencing their migratory capacity (1, 26, 47). Downregulation of chemokine receptors aids retention of PMN at sites of infection, and bacterial phagocytosis has been shown to promote this downregulation (24). A reduced migratory response of diabetic PMN toward IL-8 has been demonstrated *in vitro* (11) and is supported by the differences observed in CD11b expression on PMN from PC-T2D and ND individuals in the present study. A role for altered PMN migratory response to *B. pseudomallei* in the susceptibility of diabetic individuals to melioidosis is also supported by findings that upregulation of ICAM-1, involved in CD11b/CD18-mediated neutrophil adhesion and migration

through endothelium, is attenuated in endotoxemia in diabetic individuals compared to healthy controls (2). Murine studies have highlighted the importance of CD11b/CD18 upregulation on PMN in the early control of bacterial replication following infection (8). Within the first hours of Gram-negative bacterial infection, a rapid PMN influx is evident at sites of inflammation in immunocompetent mice (69). However, a short delay in very early PMN migration provides an opportunity for the bacterium to replicate and express virulence factors that facilitate its persistence in the host, thereby influencing disease progression (69). We propose a similar mechanism is associated with susceptibility of T2D individuals to melioidosis, whereby an initial delay in PMN migration and/or activation allows *B. pseudomallei* to multiply, establish an intracellular niche, and therefore render subsequent inflammatory responses insufficient to contain infection. Our data are supported by a recent transcriptome analysis of innate immune responses triggered after infection of *B. pseudomallei* in a chemically induced diabetic mouse model. Despite comparable bacterial loads in the first 24 h of infection, differential expression profiles for genes involved in PRR signaling pathways were demonstrated for diabetic and nondiabetic mice (13). While 16 h postinfection was the earliest time point assessed in the microarray studies of diabetic mice (13), they provide further support to our data that the presence of very early defects (<3.5 h) in the ability of phagocytes from diabetic individuals to detect and respond appropriately to *B. pseudomallei* underlies contrasting disease progression in diabetic and nondiabetic hosts. Further studies to investigate interactions between *B. pseudomallei*, PRR signaling, and the effects on leukocyte migration to sites of infection in individuals at risk of melioidosis are certainly warranted.

Our study has clear limitations. Specifically, the sample size was small and our investigations measured selected markers of the inflammatory response within 4 h of exposure to *B. pseudomallei*. Nonetheless, we believe that the data presented here identify novel differences in early cellular responses to *B. pseudomallei* between diabetic and nondiabetic individuals which are not attributed to differences in bacterial persistence. In contrast to studies examining functional responses of single leukocyte subsets, the use of a whole-blood approach enabled us to examine the responses of relevant leukocytes simultaneously, therefore more closely modeling *in vivo* inflammatory responses generated in the early stages of *B. pseudomallei* infection in diabetic and nondiabetic individuals. In addition, utilization of a whole-blood assay avoids potential bias from cellular stimulation associated with leukocyte subset isolation techniques (54). Data from this proof-of-principle study provide an immunological basis for the clinical observations observed in patients with T2D and melioidosis comorbidity (19, 62), supporting the utility of *ex vivo* whole-blood assays as a tool for evaluation of the mechanisms underlying susceptibility to *B. pseudomallei* in this at-risk population. Such studies are not possible utilizing blood from patients with diabetes and melioidosis, given that by the time they are hospitalized the infection has already been established. In addition, in most instances, patients recruited for such studies would have commenced antimicrobial therapy. Use of a whole-blood model will enable investigation of the very early interactions between human leukocytes and *B. pseudomallei* that are important in determining the outcome of infection. Given the complexity of leukocyte-leukocyte cross talk in innate immunity, use of a whole-blood model also permits measurement of inflammatory changes following *B. pseudomallei* exposure that

more closely reflect *in vivo* interactions than would be possible using specific leukocyte subsets in isolation, *in vitro*.

In summary, this is the first study to describe and characterize an *ex vivo* whole-blood model of comorbidity between diabetes and melioidosis. We have shown for the first time that contrasting inflammatory profiles for PC-T2D, WC-T2D, and ND individuals are evident as early as 3.5 h following exposure to a *B. pseudomallei* strain of low virulence, despite comparable bacterial loads. Data from our study suggest very early interactions between *B. pseudomallei* and PMN and monocytes are altered in hosts with diabetes, contributing to impaired leukocyte activation and the development of an exaggerated inflammatory response. The availability of a model that reflects clinical parameters observed in patients with T2D and melioidosis is invaluable since it will facilitate further studies of the dysregulated interactions between phagocytes and *B. pseudomallei* in the initial stages of infection in susceptible hosts and the effects of therapeutics on these interactions. This is significant, given the continuing increase in the prevalence of T2D, which could potentially place more people at risk of severe melioidosis in regions of endemicity.

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