Quantitative proteomics reveals differences in the response of neutrophils isolated from healthy or diabetic subjects to infection with capsule-variant Burkholderia thailandensis

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<td>Withatanung, Patoo; Mahidol University Faculty of Medicine Siriraj Hospital, Immunology Kurian, Dominic; The Roslin Institute, University of Edinburgh, Neurobiology Tangjittipokin, Watip; Mahidol University Faculty of Medicine Siriraj Hospital, Department of Immunology Plengvidhya, Nattachat; Mahidol University Faculty of Medicine Siriraj Hospital, Department of Immunology Titball, Richard; University of Exeter, School of Biosciences Korbsrisate, Sunee; Mahidol University Faculty of Medicine Siriraj Hospital, Immunology Stevens, Joanne; The Roslin Institute and Royal (Dick) School of Veterinary Sciences, Infection and Immunity</td>
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Quantitative proteomics reveals differences in the response of neutrophils isolated from healthy or diabetic subjects to infection with capsule-variant *Burkholderia thailandensis*

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ABSTRACT

In Thailand diabetes mellitus is the most significant risk factor for melioidosis, a severe disease caused by *Burkholderia pseudomallei*. In this study, neutrophils isolated from healthy or diabetic subjects were infected with *B. thailandensis* E555, a variant strain with a *B. pseudomallei*-like capsular polysaccharide used here as a surrogate micro-organism for *B. pseudomallei*. At two hours post-infection, neutrophil proteins were subjected to 4-plex iTRAQ-based comparative proteomic analysis. A total of 341 proteins were identified in two or more samples, of which several proteins involved in oxidative stress and inflammation were enriched in infected diabetic neutrophils. We validated this finding by demonstrating that infected diabetic neutrophils generated significantly elevated levels of pro-inflammatory cytokines TNFα, IL-6, IL-1β and IL-17 compared to healthy neutrophils. Our data also revealed that infected neutrophils from healthy or diabetic individuals undergo apoptotic cell death at distinctly different rates, with infected diabetic neutrophils showing a diminished ability to delay apoptosis and an increased likelihood of undergoing a lytic form of cell death, compared to infected neutrophils from healthy individuals. Increased expression of inflammatory proteins by infected neutrophils could contribute to the increased susceptibility to infection and inflammation in diabetic patients in melioidosis-endemic areas.

KEYWORDS: iTRAQ; Neutrophil; Diabetes mellitus; *Burkholderia thailandensis*; BTCV; Melioidosis
INTRODUCTION

Burkholderia pseudomallei, a facultative intracellular Gram-negative bacterium, is the causative agent of melioidosis. The disease is endemic in Southeast Asia and northern Australia, where mortality can be as high as 70% of infected adults in north-eastern Thailand. This bacterium has a complex intracellular lifestyle regulated by many virulence factors and has the ability to invade and replicate within host epithelial cells and phagocytic cells. B. thailandensis is genetically and phenotypically similar to B. pseudomallei, but considered to be avirulent. Despite being present in the soil of endemic areas at very high density, it has rarely been associated with clinical symptoms in humans and never associated with mortality. Recently B. thailandensis strain E555 was isolated and characterized as a novel B. thailandensis with a B. pseudomallei-like capsular polysaccharide cluster, an essential virulence factor in mammals. This strain, and others like it, are commonly referred to as B. thailandensis capsule variants (BTCVs). BTCV strains share several B. pseudomallei-like phenotypes, including colony wrinkling, resistance to complement binding and enhanced intracellular survival in macrophages when compared to non-variant B. thailandensis. Furthermore, B. thailandensis E555 generated a protective immune response in a murine model of melioidosis in a similar manner as B. pseudomallei, raising the possibility that this strain could be a potential vaccine for melioidosis. At present, little is known about the host immune response to this BTCV strain which we believe could be a very useful surrogate microbe to aid in the study of the host cell: pathogen interactions that contribute to melioidosis.

Neutrophils are an essential human innate immune cell type involved in host inflammatory processes and immune surveillance, and are often the first cells to be recruited to a site of infection. They ingest bacteria by phagocytosis and destroy pathogens by a combination of reactive oxygen species (ROS), cationic peptides and proteolytic enzymes before undergoing apoptosis and terminating the local inflammation process. Several studies
have demonstrated that neutrophils play an important role in melioidosis \(^6\)-\(^10\), through production and secretion of a variety of pro-inflammatory cytokines, for example gamma interferon (IFN-\(\gamma\)), an important cytokine for \(B.\ pseudomallei\) clearance in mice \(^11\)-\(^13\). Tumour necrosis factor (TNF-\(\alpha\)), interleukin (IL)-1\(\beta\) and IL-6 are also important for bacterial clearance in murine models of acute and chronic melioidosis \(^14\). Furthermore, neutrophils from healthy volunteers have also been shown to destroy \(B.\ pseudomallei\) through a process of extrusion of cellular contents including DNA, proteases and antimicrobial proteins in structures known as Neutrophil Extracellular Traps (NETs) \(^15\), and through macroautophagy \(^16\).

Diabetes mellitus (DM) is the most significant comorbidity risk factor for patients with melioidosis in Thailand. Up to 60% of melioidosis patients have pre-existing type 2 DM with acute rather than chronic infection \(^17\). Glucose and glutamine play key roles in neutrophil function \(^18\) and the hyperglycaemia status of DM patients has been reported to significantly correlate with neutrophil dysfunction \(^19\), \(^20\). The neutrophils of diabetic melioidosis patients demonstrate significant defects in migration, phagocytosis, ability to delay apoptosis \(^21\) and reduced NET formation upon \(B.\ pseudomallei\) infection \(^15\), when compared with neutrophils isolated from healthy subjects.

To better understand the role of neutrophils in melioidosis, we have studied the proteome of neutrophils isolated from healthy donors and diabetic patients in response to the \(B.\ pseudomallei\)-like BTCV strain E555 using iTRAQ quantitative proteomics. We identified 341 proteins in two or more samples and were able to calculate ratios for these proteins and demonstrate significant differences in the protein profiles of neutrophils from healthy and diabetic subjects.
EXPERIMENTAL SECTION

Human subjects and ethics statement

This study was reviewed and approved by the Siriraj Ethics Committee for Human Research Protection Unit and written informed consent was obtained from all volunteers. Diabetic volunteers (n=4) had pre-existing type 2 diabetes (T2DM) with random blood glucose levels ≥200 mg/dl, fasting blood glucose levels ≥126 mg/dl, oral glucose tolerance test ≥200 mg/dl, glycosylated haemoglobin A1c (HbA1c) levels ≥6.5%. Healthy volunteers (n=4) had normal blood counts, normal fasting blood glucose levels and normal glycosylated haemoglobin levels.

Blood sample collection and neutrophil isolation

Human whole venous blood was collected from 4 healthy or diabetes mellitus volunteers with preservative-free lithium heparin as an anticoagulant. In brief, 5 ml of human whole venous blood was carefully layered over 5 ml of Polymorphprep reagent (13.8% (w/v) sodium diatrizoate, 8.0% (w/v) polysaccharide, density: 1.113 g/ml). After centrifugation at 600x g for 40 min at 20°C, the PMNs (Figure S1) were collected before washing with PBS. After centrifugation at 400x g for 5 min at 20°C, the supernatant was discarded and contaminating RBCs were lysed with RBC lysis buffer. After centrifugation, the cell pellet was collected and washed with PBS before centrifugation at 400x g for 5 min at 20°C. The pellet was collected and re-suspended in RPMI1640 media containing 10% FBS (Thermo Scientific, MA) to obtain neutrophils with a routine viability of >96% (determined by 0.4% Trypan blue exclusion) and purity of >97%, (determined by microscopy and flow cytometry).
Neutrophil infection

Human healthy or diabetic neutrophils were seeded to obtain $5 \times 10^5$ cells in a 24-well plate before infecting with *B. thailandensis* capsule-variant strain E555 (at mid-logarithmic phase of growth in LB broth) at an MOI of 5, with uninfected cells as negative controls. After incubation at 37°C with 5% CO$_2$ for 30 min (T0), infected cells were washed with PBS before adding RPMI1640 containing 10% FBS and 250 µg/mL kanamycin to kill the extracellular bacteria. After incubation at 37°C with 5% CO$_2$ for the indicated time points, infected neutrophils were washed and centrifuged at 500x$g$ for 2 min. Supernatants were collected for cytokine analysis and for plating on LB agar to confirm a lack of viable extracellular bacteria. The cell pellet was collected and lysed using 1% Triton X-100 in PBS to enumerate the number of intracellular bacteria, or to isolate the total protein fractions for iTRAQ labelling and MS analysis.

Protein extraction, digestion, iTRAQ labelling and LC-MS

About $1 \times 10^6$ neutrophils were mixed with 250µl 20mM Tris-pH 7.5, 200mM NaCl, and 1% (w/v) octylthioglucoside before incubation for 1 h at room temperature with gentle agitation. The samples were centrifuged at 16,000x$g$ for 20 min at 4°C and the supernatant containing the extracted protein collected. Protein samples were acetone precipitated, suspended in 100mM triethylammonium bicarbonate (TEAB) buffer containing 0.1% SDS and protein concentration determined. Aliquots containing 10µg of protein from each condition were reduced with 5mM tris (2-carboxyethyl) phosphine (TCEP) for 1 hour at 60°C and alkylated with 10mM methyl methanethiosulfonate (MMTS) for 30 min in the dark at room temperature. In-solution digestion was performed with sequencing-grade trypsin (Promega Corporation) in a 1:20 mass ratio at 37°C. The resulting tryptic peptides were labelled with iTRAQ reagents (4-Plex system) according to the manufacturer’s instructions (Sciex,
The 114 and 115 iTRAQ tags were used for the samples from normal uninfected (NM-) and infected (NM+) samples and the 116 and 117 tags were used for diabetic uninfected (DM-) and infected (DM+) samples, respectively. Briefly, iTRAQ labelling reagents were dissolved in 70µL of ethanol and the contents were transferred to vials containing respective tryptic peptides. After 2 hours of incubation at room temperature, the reaction was quenched by adding an equal volume of water. Differentially labelled peptides were mixed and dried under vacuum.

The pooled iTRAQ labelled peptides were fractionated by basic pH reversed-phase chromatography on an XBridge BEH C\textsubscript{18} (1 x 50 mm) column using a Dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific, UK). Briefly, the solvent gradients of buffer A (95% H\textsubscript{2}O and 5% ACN, pH 10,) and buffer B (5% H\textsubscript{2}O and 95% ACN, pH 10) were as follows: 3%–5% B, 5 min; 5%–32% B, 25 min; 32%–95% B, 5 min; 95% B, 5 min; and 95%–3% B, 2 min. The LC flow rate was set at 0.2 mL/min and monitored at 280 nm. The eluent was collected every 1 min. Fractions were desalted on Stage-Tip columns according to Rappsilber et al.\textsuperscript{22}.

Peptide fractions were loaded on to an Acclaim PepMap100, C\textsubscript{18}, 100 Å, 75\textmu m (internal diameter) × 15cm column using a Dionex UltiMate RSLCnano System (ThermoFisher Scientific, UK). The peptides eluted by reversed-phase chromatography were analysed by a micrOTOF-Q II mass spectrometer (Bruker Daltonics, Bremen, Germany) which was operated in data-dependent mode, automatically switching between MS and MS/MS mode. The m/z values of tryptic peptides were measured using a MS scan (300-2000 m/z), followed by MS/MS scans of the six most intense peaks. Rolling collision energy for fragmentation was selected based on the precursor ion mass and a dynamic exclusion was applied for 60 seconds.

Raw spectral data were processed by DataAnalysis software (Bruker Daltonics, Bremen, Germany) and the resulting peaklists were searched using Mascot 2.4 server (Matrix Science,
UK) against the Uniprot Human sequence database containing 89,796 entries. Mass tolerance on peptide precursor ions was fixed at 25ppm and on fragment ions at 0.06Da. The peptide charge states were set as 2+ and 3+. iTRAQ modified N-terminal residue, iTRAQ modified lysine and methylthio modified cysteine were set as fixed modifications, while oxidation of methionine and iTRAQ modified tyrosine were set as variable modifications. The peptides were filtered to a FDR of lower than 1%, estimated using the target-decoy search strategy. iTRAQ quantification was performed by using WARPLC plugin on ProteinScape 3.1 software (Bruker Daltonics, Bremen, Germany) to extract reporter ion intensities of every MS/MS spectra with a requirement of two or more peptides. Peptide ratios were normalized based on setting overall peptide median ratio at one, which corrects for unequal protein sampling, and a coefficient of variability of peptide ratios were also determined for each quantified proteins.

Arbitrary cut-off values of iTRAQ ratios of ≥1.2 and of ≤0.8 were applied to the data to establish lists of differentially expressed proteins. Molecular functions and biological processes were inferred using a variety of online tools including Reactome (reactome.org).

**SDS-PAGE and Western blot analysis**

Approximately 50 µg denatured protein samples were separated by 10-12% SDS-PAGE before transfer to a nitrocellulose membrane. Membranes were blocked with 5% skimmed milk protein before washing with PBST (PBS with 0.5% Tween-20). The membranes were then probed with anti-myeloperoxidase [2C7] (#ab25989, Abcam, UK), anti-S100A9 [D5O6O] (#72590, Cell signalling technology, USA) or anti-β-actin [D6A8] (# 8457, Cell signalling technology, USA) primary antibodies at a dilution of 1:1,000 before detecting using the relevant species HRP-conjugated secondary antibodies at a dilution of 1: 10,000. Bands were detected using Novex ECL Chemiluminescent Substrate Reagent Kit (ThermoFisher
Scientific, USA). Images were captured using GeneSnap software version 7.02 (SynGene, USA).

Confocal microscopy

Neutrophils were seeded into a 6-well plate containing glass cover slips before infecting with *B. thailandensis* E555 at an MOI of 5 for 2 h as described above. Uninfected or *B. thailandensis* E555-infected neutrophils were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton-X 100 in PBS. Cells were then blocked with 3% BSA before incubating with anti-myeloperoxidase [2C7] (1:200; #ab25989, Abcam, UK), or anti- *B. thailandensis* E555 (1:100; kindly provided by Dr. Ganjana Lertmemongkolchai, Khon Kaen University, Thailand). Cells were then incubated with goat anti-mouse<br>568 (1:500; #A-11004, ThermoFisher Scientific, USA) and goat anti-rabbit 488 (1:500; #A27034, ThermoFisher Scientific, USA) secondary antibodies and counter-stained with 4′-6-diamidino-2-phenylindole (DAPI; 1:500; Molecular probes, USA). After incubation, cover slips were mounted and analysed by laser scanning confocal microscopy (LSM800, Carl Zeiss, Switzerland).

Cytokine measurement by ELISA

Supernatants from both uninfected and infected cells were collected and filtered through 0.22μm filters to remove any cell debris or intact viable bacteria. The concentrations of human IL-1β, IL-6, IL-8, TNF-α, IL-10 and IL-17A were determined using commercially available ELISA kits, according to the manufacturer’s instructions. Samples from three independent experiments (n=3 biological repeats), each with three intra-experiment technical replicates were assayed.
Neutrophil apoptosis and LDH release assays

Neutrophil apoptosis was determined by flow cytometry using annexin V488/Dead cell apoptosis kit (ThermoFisher Scientific, USA), according to the manufacturer’s instructions. About 5x10^5 neutrophils were collected after 2 and 24 h post-infection before re-suspending with annexin-binding buffer. Then, 5μL of annexin V488 and 1μl of 100 μg/ml propidium iodine (PI) were added to each tube. After 15 min incubation, 400μl of annexin-binding buffer was added and the stained cells analyzed by flow cytometry (FACSCalibur, BD Biosciences, USA). Extracellular lactate dehydrogenase (LDH) released from uninfected or infected neutrophils were quantitatively determined in supernatants using a commercial LDH cytotoxicity assay kit (ThermoFisher Scientific, USA), following the manufacturer’s instructions. Samples from three independent experiments (n=3 biological repeats), each with three intra-experiment technical replicates were assayed for each cytokine.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad, CA, USA). The unpaired Students t-test was applied for comparing the means of two independent sample groups. P-values less than 0.05 were considered statistically significant (*p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001, ns; not significant; p-value > 0.05).
RESULTS & DISCUSSION

Bactericidal activity of neutrophils isolated from healthy and diabetic subjects

There are few reports directly comparing the bactericidal activity of neutrophils from healthy and diabetic donors challenged in vitro with *Burkholderia pseudomallei*, and none that compare the responses of such cells to *B. thailandensis* or *B. thailandensis* capsule-variant (BTCV) strains. In this study, BTCV strain E555 was used as a surrogate model organism for *B. pseudomallei* due to its enhanced survival inside macrophage-like phagocytic cells in a manner dependent on the capsular polysaccharide gene cluster when compared to non-variant *B. thailandensis* \(^4\), and also because it can be handled at lower containment with lower health risk to laboratory workers. To compare the responses of neutrophils to infection, we carefully screened potential donors and selected diabetic subjects with relatively poor glycemic control who were not medicated with glibenclamide. We excluded glibenclamide-treated patients based on the fact that Kewcharoenwong *et al.* \(^{23}\) had previously demonstrated that glibenclamide drug treatment in DM patients significantly affected several neutrophil functions in response to *B. pseudomallei* infection. Fresh human whole venous blood were collected from either 4 healthy or 4 DM subjects and pooled together to reduce the variability between individual subjects such as age, weight and drug treatment, before isolation of the neutrophils.

Neutrophils were isolated from the blood of donors as described in the experimental section. Routinely, cells of more than 95% viability and 97% purity (as assessed by microscopy and flow cytometry analysis) were obtained (Figure S1). The neutrophils were first infected with *B. thailandensis* capsule variant (BTCV) strain E555 and the number of viable intracellular bacteria recovered at several time points thereafter. The number of viable bacteria recovered at 30 minutes, 2, 4 and 6 hours post-infection were calculated as a percentage of the initial inoculum used to infect the cells, and the data plotted. At 30 minutes post-infection (T0), a significant difference in the number of viable bacteria recovered from cells from healthy
subjects compared to cells isolated from diabetic donors was detected (Figure 1). This indicated that the diabetic neutrophils displayed a lower level of phagocytosis, as has been previously described \(^{21, 24}\). Despite a reduced level of initial bacterial uptake (T0), the neutrophils from diabetic volunteers displayed significantly higher levels of bactericidal activity when compared to neutrophils from healthy volunteers at both 2 and 4 hours post-infection (Figure 1). Although by 6 hours post-infection, no significant difference in bactericidal activity between neutrophils from healthy or diabetic donors could be observed (Figure 1). These results are in accordance with those described by Chanchamroen et al. who demonstrated that whilst neutrophils from diabetic patients are less phagocytic, they show increased rates of bactericidal activity against \textit{B. pseudomallei} over time \(^{21}\).
Figure 1. Comparison of the bactericidal activity of neutrophils isolated from healthy (NM) and diabetic (DM) volunteers. Neutrophils were infected with *B. thailandensis* E555 at an MOI of 5. Cells were lysed at 30 minutes (T0), 2 (T2), 4 (T4) and 6 (T6) hours post-infection for enumeration of viable bacteria. The number of intracellular live bacteria was expressed as a percentage of the initial inoculum using the equation \([(\text{number of intracellular viable bacteria at a single time point} / \text{number of bacteria in the inoculum}) \times 100]\). Each bar indicates the mean ± SEM from data obtained from 3 independent experiments (n=3 three biological replicates). An unpaired Students *t*-test was used to compare the numbers of intracellular bacteria recovered from healthy neutrophils to those recovered from diabetic neutrophils at each time point, where * indicates p-value ≤0.05.
Characterisation of the neutrophil proteomes from healthy and diabetic donors

Neutrophils obtained from both healthy and diabetic donors were next infected with *B. thailandensis* E555 at an MOI of 5 for a total of 2 hours, before lysis and extraction of protein as described in the experimental section. Uninfected neutrophils treated under the same experimental conditions were lysed as control samples. Total protein samples of uninfected healthy neutrophils (NM-), *B. thailandensis* E555-infected healthy neutrophils (NM+), uninfected diabetic neutrophils (DM-) and *B. thailandensis* E555-infected diabetic neutrophils (DM+) were then subjected to 4-plex iTRAQ comparative proteomics analysis as outlined in Figure S2 and described in detail in the experimental section. Following LC-MS/MS, the raw spectral data were processed and protein identifications assigned. A total of 341 proteins were identified in two or more samples, allowing relative abundance ratios (fold changes) to be calculated for these proteins. Arbitrary cut-off values of >1.2 and <0.8 were applied to identify candidate proteins exhibiting increased or decreased expression respectively (Supplemental Table S1).

Comparison of the levels of proteins expressed by uninfected neutrophils (DM-/NM-) identified 86 proteins with a significant increase in expression in resting neutrophils from healthy donors, compared with neutrophils from diabetic donors. Conversely, 94 different proteins demonstrated an increased expression in uninfected neutrophils from diabetics compared to healthy donors (Table S1). To our knowledge, this is the first description of the proteome of neutrophils from diabetic patients. Pathway analysis of the proteins with increased expression in normal or diabetic neutrophils using the online Reactome resource, highlighted a large number of conserved pathways between healthy and diabetic subjects, as well as a few distinct pathways. Amongst the conserved pathways were those involved in antigen processing and presentation, interferon and cytokine signalling, and neutrophil degranulation. Perhaps unsurprisingly, two pathways that were unique to the normal neutrophils were
‘gluconeogenesis’ and ‘glucose metabolism’. Of significance when analysing the proteins with increased expression in diabetic cells, were proteins mapping to the ‘antimicrobial peptides’ pathway. These proteins were Eosinophil cationic protein, Protein S100-A8, Protein S100-A9, Cathepsin G, Myeloblastin, Neutrophil defensin 1, Peptidoglycan recognition protein 1 and Lactotransferrin.

Within the lists of proteins that are significantly increased in either the resting neutrophils from healthy or diabetic donors, we noted that cells from diabetic patients over-expressed calpain, a potent activator of apoptosis in neutrophils. Conversely, cells from healthy subjects overexpressed calpastatin, an inhibitor of apoptosis in neutrophils, suggesting that cells from diabetics may be primed to undergo cell death or less able to delay spontaneous apoptosis in response to pathogens. In addition, we also noted the overexpression of a number of proteins in diabetic neutrophils involved in NET formation, a neutrophil specific mechanism of trapping and killing extracellular pathogens involving extrusion of cellular contents such as DNA, proteases and antimicrobial proteins, many of which are potent inflammatory mediators. Comparison with previous proteomic studies on the composition of NETs indicated that 13 NET-associated proteins were identified in our study as significantly overexpressed in diabetic resting neutrophils. Resistin, originally identified as an insulin resistance factor and more recently shown to be a potent activator of NETs, was also significantly overexpressed in neutrophils from diabetic volunteers.

**Comparison of the proteome of neutrophils isolated from healthy and diabetic volunteers infected in vitro with BTCV E555**

We further analysed our dataset by comparing the relative abundance of proteins in infected diabetic and infected healthy neutrophils (DM+/NM+, Table S1). Of the 316 proteins detected in both samples, 69 proteins demonstrated a significant increase in expression in
infected healthy neutrophils and 116 unique proteins were overexpressed in infected neutrophils from diabetics (Figure 2). The 30 proteins displaying the most extensive fold change in response to infection with this intracellular bacterium are listed in Table 1. This is the first proteomic comparison of the response of neutrophils from healthy and diabetic individuals. Mapping of these proteins onto molecular pathways using Reactome, confirmed that both infected healthy and diabetic neutrophils responded by increasing expression of proteins associated with the ‘innate immune system’ and ‘neutrophil degranulation’, with significantly more proteins mapping to these pathways from the infected diabetic cells. An increased number of proteins associated with innate immunity and neutrophil degranulation in infected diabetic neutrophils is suggestive of an increased inflammatory response by these cells compared to infected healthy neutrophils. Excessive neutrophil degranulation is a common cause of many inflammatory diseases, such as severe asphyxic episodes of asthma, acute lung injury, rheumatoid arthritis, and septic shock 

Further evidence for an increased inflammatory response of diabetic neutrophils upon infection, comes from the fact that a significant number of proteins from infected diabetic cells mapped to pathways involved in interferon and cytokine signalling, antigen presentation and antimicrobial peptides. Conversely, pathways mapping to the proteins increased in healthy infected cells were limited to those involved in ‘calcineurin activation of NFAT’, glycolysis and gluconeogenesis, ‘IL12 and JAK-STAT signalling’.
Figure 2. Venn diagram illustrating the numbers of common and unique proteins with increased expression upon infection of neutrophils isolated from either healthy or diabetic volunteers.
Table 1: Top 30 proteins with highest fold change upon infection of neutrophils from healthy or diabetic volunteers. Proteins with increased abundance upon infection of neutrophils from healthy volunteers (NM) are shown on the left hand side of the table (Fold change DM+/NM+ = ≤0.8). Proteins with increased abundance upon infection of neutrophils from diabetic volunteers (DM) are shown on the right hand side of the table (Fold change DM+/NM+ = ≥1.2).

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<td>Isoform 3 of Leucine-rich repeat flightless-interacting protein 1</td>
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<td>Synaptic vesicle membrane protein VAT-1 homolog</td>
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<td>0.61</td>
<td>P05109</td>
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Several proteins involved in oxidative stress were overexpressed in infected diabetic cells including myeloperoxidase and eosinophil peroxidase, which displayed 8.6 and 6.7 fold more protein in infected diabetic cells compared to infected healthy cells respectively (Table 1). Interestingly, myeloperoxidase was also expressed at higher levels in uninfected diabetic cells compared to uninfected healthy cells, with a relative abundance ratio of 1.7. Similarly, we noted that the proteins S100-A8 and S100-A9, which together form the pro-inflammatory and anti-microbial complex calprotectin, were significantly increased in infected diabetic cells compared to infected neutrophils from healthy volunteers, with a relative abundance ratio of 2.3 and 1.7 respectively. We chose to validate these findings by western blotting independently prepared samples of uninfected neutrophils from healthy donors (NM-), infected healthy neutrophils (NM+), uninfected neutrophils from diabetic donors (DM-) and infected diabetic neutrophils (Figure 3). As indicated by the proteomic data, myeloperoxidase could be detected in all samples, with an increased level of expression in resting neutrophils from diabetic donors compared to resting neutrophils from healthy donors. Myeloperoxidase expression increased upon infection, with significantly higher levels in the infected diabetic cells (Figure 3). This data correlates with a previous study by Morris et al. 32, who showed that myeloperoxidase release is significantly higher in whole blood of Type 2 DM patients than healthy controls upon B. pseudomallei infection. Excessive release of myeloperoxidase has been associated with severe local tissue inflammation through the generation of reactive halogenated and nitrated compounds in vivo 33. Similarly, we confirmed that expression of the inflammation-associated S100-A9 protein was also increased upon infection, with highest expression levels in infected diabetic cells (Figure 3). Increased expression of myeloperoxidase was also demonstrated in cells in vitro by immunostaining and confocal microscopy of uninfected and infected neutrophils (Figure 4). These findings support our hypothesis that diabetic neutrophils abundantly express inflammatory and oxidative stress mediators upon infection.
Figure 3. Western blot analysis of Myeloperoxidase and S100-A9 in uninfected and *B. thailandensis* E555-infected healthy and diabetic neutrophils. Cells were incubated with media (uninfected) or with *B. thailandensis* E555 at an MOI of 5 for 2 hours. After lysis, separation by SDS-PAGE and transfer to nitrocellulose, the neutrophil proteins were detected with antibodies specific to myeloperoxidase (MPO) (upper panel), S100A9 (middle panel) or β-actin blotting (lower panel), which was used as a loading control.
Figure 4. Cellular localisation of Myeloperoxidase in uninfected and *B. thailandensis* E555-infected healthy or diabetic neutrophils. Neutrophils were incubated in media or with *B. thailandensis* E555 at an MOI of 5 for 2 hours. Cells were fixed and permeabilized before immunostaining with mouse anti-MPO and rabbit anti-LPS primary antibodies. Cells were then probed with goat-anti mouse and goat anti-rabbit to illustrate Myeloperoxidase localisation in red and bacteria in green. Host cell DNA was counter-stained with DAPI (blue). Each panel represents a composite image generated from several serial optical sections, using data captured with identical laser settings. Scale bar = 10μm.
Myeloperoxidase and calprotectin are also molecules extruded by neutrophils in NETs upon infection. We detected 18 NET-associated proteins \(^{26-28}\) that were overexpressed in diabetic neutrophils compared to healthy neutrophils in response to infection (Table 2), although we did not investigate NET formation in this study. Whilst our data conflicts with a previous report that suggested that neutrophils from diabetic patients were less able to produce NETs in response to infection with *B. pseudomallei* \(^{15}\), it is in agreement with a second study illustrating that NET markers are significantly elevated in the plasma of DM patients compared to healthy controls, and that these markers are further increased in DM patients with melioidosis \(^{34}\). However, an alternative hypothesis could be that the biological difference in neutrophil responses lies in the ability of the cells to undergo degranulation, since many of the proteins in Table 2 and within the overall list of 116 proteins overexpressed by infected neutrophils from diabetic donors are associated with neutrophil degranulation and are potent inflammatory mediators.
Table 2. NET-associated proteins with increased expression in neutrophils from diabetic subjects following infection. Fold change refers to the abundance ratio in comparison with neutrophils isolated from healthy volunteers upon infection with *B. thailandensis* E555.

<table>
<thead>
<tr>
<th>Accession no.</th>
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<th>Fold change</th>
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Comparison of neutrophil cytokine production following infection

The data presented in this study, particularly the increased levels of proinflammatory mediators in DM neutrophils both before and after infection, were suggestive of an increased inflammatory response in neutrophils from diabetic subjects when challenged in vitro with BTCV E555. Indeed others have demonstrated that *B. pseudomallei* can induce elevated levels of the pro-inflammatory cytokines TNFα, IL-6, IL-8 and IL-12p70 in whole blood of diabetic subjects compared to healthy subjects. We assessed the levels of TNFα, IL-6, IL-1β and IL-10 in supernatants of neutrophils treated with media or infected with BTCV E555 for 2 and 24 hours by ELISA (Figure 5). At 2 hours post-infection, TNFα, IL-6 and IL-1β were elevated in the supernatants of both diabetic and healthy infected neutrophils (Figure 5A). Furthermore, TNFα, IL-6 and IL-1β levels were significantly higher in infected diabetic neutrophils compared to infected healthy neutrophils, indicating that these cells were mounting a greater pro-inflammatory response. By 24 hours post-infection, only IL-1β remained significantly higher in the infected diabetic cells compared to the infected healthy cells (Figure 5B).

IL-10 levels remained largely unchanged over time in control uninfected neutrophils, but did increase upon infection (Figure 5). As a key anti-inflammatory cytokine involved in dampening down inflammatory responses following successful control of pathogens, IL-10 levels would be expected to increase at later time points of infection. Indeed, this was the case when comparing the IL-10 secretion at 2 hours post-infection of healthy neutrophils compared to 24 hours post-infection. IL-10 secretion was 7-fold higher in infected NM neutrophils at 2 hours post-infection and 12-fold higher at 24 hours post-infection. However, infection of diabetic neutrophils resulted in lower levels of IL-10 at both 2 and 24 hours post-infection compared to infection of NM cells. Upon infection of DM neutrophils, a 4-fold and 5-fold increase in IL-10 was detected at 2 and 24 hours post-infection respectively, implying that these
cells lack the ability to regulate and dampen down the pro-inflammatory cytokine response following infection.
Figure 5. Cytokine secretion by uninfected and E555-infected neutrophils from healthy and diabetic subjects. Neutrophils from healthy (NM) or diabetic (DM) volunteers were incubated with media or infected with *B. thailandensis* E555 at an MOI of 5 for 2 hours (T2) (A) and 24 hours (T24) (B). Secretion of IL-10, TNFα, IL-6 and IL-1β in cell supernatants were detected by ELISA. An unpaired Students t-test was used to compare the means of two independent sample groups. Each bar indicates the mean ± SEM for data obtained from three independent experiments (n=3 biological replicates), where * = p-value ≤0.05, ** = p-value ≤0.01 and *** = p-value ≤0.001.
Neutrophils are a significant source of the pro-inflammatory cytokine IL-17, a cytokine with autocrine function which increases expression of other pro-inflammatory cytokines, proteases and reactive oxygen species, upon LPS stimulation. IL-17 has been linked to effective anti-fungal responses but also to chronic inflammatory conditions such as rheumatoid arthritis and psoriasis, and has been shown to be elevated in the circulation of Type 2 diabetes patients. Here we studied the secretion of IL-17A in neutrophils from healthy and diabetic subjects treated with media or BTCV E555 for 2 and 24 hours. Cells from healthy volunteers failed to stimulate an appreciable level of secreted IL-17, regardless of infection status (Figure 6). Conversely, uninfected neutrophils from diabetic volunteers secreted readily detectable levels of IL-17 in the absence of infection (between 50-60pg/ml at both 2 and 24 hours) (Figure 6). Significantly, levels of secreted IL-17 increased further upon infection of the diabetic neutrophils, and over time.
Figure 6. Secretion of IL-17 by uninfected and E555-infected neutrophils from healthy and diabetic subjects. Neutrophils from healthy (NM) or diabetic (DM) volunteers were incubated with media or infected with *B. thailandensis* E555 at an MOI of 5 for 2 hours (T2) (A) and 24 hours (T24) (B). Secretion of IL-17 in cell supernatants was detected by ELISA. Each bar indicates the mean ± SEM for data obtained from three independent experiments (n=3 biological replicates). An unpaired Students *t*-test was used to compare the means of two independent sample groups, where ** indicates p-value ≤0.01.
Comparison of rates of apoptosis in resting and infected neutrophils from healthy and diabetic donors

In our initial analysis of the proteomic dataset we noticed that neutrophils from healthy donors overexpressed calpastatin (an inhibitor of apoptosis in neutrophils) \(^{25}\) whilst expressing a lower level of calpain in comparison to neutrophils from diabetic subjects (Table S1). Our pro-inflammatory cytokine data demonstrated that a significantly higher level of TNF\(\alpha\), a cytokine with a role in induction of apoptosis, was secreted by infected diabetic cells at significantly higher levels a 2 hours post-infection. Previously, Chanchamroen et al. \(^{21}\) demonstrated that \(B.\) pseudomallei-infected healthy neutrophils were able to delay spontaneous apoptosis, whilst infected diabetic neutrophils demonstrated significantly higher levels of programmed cell death at 24 hours post-infection. With these data in mind, we assessed the induction of apoptosis in neutrophils from healthy and diabetic subjects following incubation with media or BTCV E555 for 2 and 24 hours (Figure 7). Cells were incubated with Annexin V\(^{488}\) and propidium iodide (PI), and assessed by flow cytometry (Figure 7C). Annexin V binds to apoptotic cell membranes, whereas the cell-impermeable DNA-binding PI will only enter cells undergoing secondary necrosis or necrosis. As previously shown by Chanchamroen et al. \(^{21}\), in our experiments neutrophils from healthy donors were significantly delayed in apoptosis following infection with BTCV for 2 hours (Figure 7A & 7C) and 24 hours (Figure 7B & 7C). Whilst uninfected neutrophils from diabetic donors displayed similar levels of spontaneous apoptosis over the two time points studied as the uninfected neutrophils from healthy donors, infection of the neutrophils from diabetics resulted in a less marked but significant delay in apoptosis at 2 hours post-infection (Figure 7A). This was accompanied by a small but significant increase in the number of cells that were both Annexin V and PI positive at 2 hours post-infection (labelled ‘late apoptosis’ in the figure), indicating that a proportion of cells had completed the apoptotic process and were in the stage of late apoptosis/ secondary necrosis, or...
possibly undergoing cell death through necrosis or an alternative form of cell death involving membrane disintegration.
Figure 7. Assessment of apoptosis of uninfected and *B. thailandensis* E555-infected neutrophils from healthy or diabetic individuals. Neutrophils were treated with media or infected with *B. thailandensis* E555 at an MOI of 5 for 2 hours (T2, A) and 24 hours (T24, B). Cells were stained with annexin V<sup>488</sup> and propidium iodine (PI) before flow cytometry analysis. Each bar indicates the mean ± SEM for data obtained from three independent experiments (n=3 biological replicates). An unpaired Students *t*-test was used to compare the means of two independent sample groups, where * = p-value ≤0.05, ** = p-value ≤0.01 and *** = p-value ≤0.001. Representative plots of data obtained from NM neutrophils before and after infection at 2 (T2) and 24 (T24) hours post-infection are shown in C.

One classic feature of cell death by necrosis that distinguishes this process from apoptosis, is a loss of plasma and nuclear membrane integrity. This can be measured by assessing the levels of Lactate dehydrogenase in cell supernatants, a cytoplasmic enzyme which is only present in the extracellular media upon disintegration of the plasma membrane. Infected healthy neutrophils display decreased levels of cell lysis compared to the uninfected control cells, at both 2 and 24 hours post-infection (Figure 8). Conversely, infection of neutrophils isolated from diabetic donors displayed a consistent and significant increase in cell lysis at both 2 and 24 hours post-infection (Figure 8). This data suggests that neutrophils from diabetic subjects are more likely than neutrophils from healthy donors to undergo a form of lytic cell death, such as necrosis, upon infection with BTCV.
Figure 8. Assessment of cell integrity of uninfected and *B. thailandensis* E555-infected neutrophils from healthy and diabetic individuals. Neutrophils were treated with media or infected with *B. thailandensis* E555 at an MOI of 5 for 2 hours (T2) and 24 hours (T24). Cell supernatants were harvested and assayed for extracellular lactate dehydrogenase (LDH). Each bar indicates the mean ± SEM for data obtained from three independent experiments (n=3 biological replicates). An unpaired Students *t*-test was used to compare the means of two independent sample groups, where * = p-value ≤0.05, ** = p-value ≤0.01 and *** = p-value ≤0.001.
CONCLUSION

Here we describe the first quantitative proteomic analysis of neutrophils from patients with Type 2 diabetes (Diabetes mellitus) in comparison with neutrophils from healthy volunteers. Furthermore, this study is the first to compare the response of these neutrophils upon infection with the Gram negative bacterium *B. thailandensis* capsule-variant strain E555, used here as a surrogate organism for *B. pseudomallei*, the causative agent of melioidosis. Our data provide important insights into the differential response of neutrophils from healthy and diabetic subjects upon infection, where neutrophils from diabetic subjects produce significantly more inflammatory mediators, proteins involved in oxidative stress and display an increased rate of cell death associated with loss of plasma membrane integrity. Overall our study presents novel data that will aid in a better understanding of the host: pathogen interactions involved in the life-threatening disease melioidosis, particularly in high risk patients.
ASSOCIATED CONTENT

Data

The Mass spectrometry data have been deposited to the MassIVE archive (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp) with the data set identifier MSV000083765.

Supporting information

Table S1. List of proteins detected and their calculated abundance ratios.

Figure S1. Neutrophil isolation.

Figure S2. Diagram illustrating the proteomic workflow.

Author contributions

Conceived and designed experiments: JMS, SK, PW, and RWT. Performed the experiments: PW, DK. Analyzed the data: PW, JMS, DK. Contributed reagents/materials/analysis tools: JMS, RWT, WT, and NP. Wrote the paper: PW, JMS, SK.

Conflict of interests

The authors declare no competing financial interest.

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ABBREVIATIONS

CFU; colony forming unit, h; hour(s), IFN; interferon, iTRAQ; isobaric tags for relative and absolute quantitation, IL; interleukin, LC-MS/MS; liquid chromatography (LC) with mass spectrometry, PBS; phosphate-buffered saline, SDS-PAGE; sodium dodecyl sulphate polyacrylamide gel electrophoresis, TNF; tumour necrosis factor
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against *Burkholderia pseudomallei* and are influenced by bacterial and host factors.


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Figure 1

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Figure 2

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