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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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3 **Quantitative proteomics reveals differences in the response of neutrophils**
4 **isolated from healthy or diabetic subjects to infection with capsule-variant**
5 ***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 TNF α , IL-6, IL-1 β 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 mortality¹⁻³. Recently *B. thailandensis* strain E555 was isolated and characterized as a novel *B. thailandensis* with a *B. pseudomallei*-like capsular polysaccharide cluster⁴, 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*⁴. 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 melioidosis⁵. 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

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2
3 have demonstrated that neutrophils play an important role in melioidosis ⁶⁻¹⁰, through
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5 production and secretion of a variety of pro-inflammatory cytokines, for example gamma
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7 interferon (IFN- γ), an important cytokine for *B. pseudomallei* clearance in mice ¹¹⁻¹³. Tumour
8
9 necrosis factor (TNF- α), interleukin (IL)-1 β and IL-6 are also important for bacterial clearance
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11 in murine models of acute and chronic melioidosis ¹⁴. Furthermore, neutrophils from healthy
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13 volunteers have also been shown to destroy *B. pseudomallei* through a process of extrusion of
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15 cellular contents including DNA, proteases and antimicrobial proteins in structures known as
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17 Neutrophil Extracellular Traps (NETs) ¹⁵, and through macroautophagy ¹⁶.

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

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40 To better understand the role of neutrophils in melioidosis, we have studied the proteome
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42 of neutrophils isolated from healthy donors and diabetic patients in response to the *B.*
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44 *pseudomallei*-like BTCV strain E555 using iTRAQ quantitative proteomics. We identified 341
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46 proteins in two or more samples and were able to calculate ratios for these proteins and
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48 demonstrate significant differences in the protein profiles of neutrophils from healthy and
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50 diabetic subjects.
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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 $\geq 6.5\%$. 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 5×10^5 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% CO₂ 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% CO₂ 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 1×10^6 neutrophils were mixed with 250 µl 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 10 µg 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,

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3 Warrington, UK). The 114 and 115 iTRAQ tags were used for the samples from normal
4 uninfected (NM-) and infected (NM+) samples and the 116 and 117 tags were used for diabetic
5 uninfected (DM-) and infected (DM+) samples, respectively. Briefly, iTRAQ labelling reagents
6 were dissolved in 70 μ L of ethanol and the contents were transferred to vials containing
7 respective tryptic peptides. After 2 hours of incubation at room temperature, the reaction was
8 quenched by adding an equal volume of water. Differentially labelled peptides were mixed and
9 dried under vacuum.
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19 The pooled iTRAQ labelled peptides were fractionated by basic pH reversed-phase
20 chromatography on an XBridge BEH C₁₈ (1 x 50 mm) column using a Dionex Ultimate 3000
21 UHPLC system (ThermoFisher Scientific, UK). Briefly, the solvent gradients of buffer A (95%
22 H₂O and 5% ACN, pH 10,) and buffer B (5% H₂O and 95% ACN, pH 10) were as follows:
23 3%–5% B, 5 min; 5%–32% B, 25 min; 32%–95% B, 5 min; 95% B, 5 min; and 95%–3% B, 2
24 min. The LC flow rate was set at 0.2 mL/min and monitored at 280 nm. The eluent was
25 collected every 1 min. Fractions were desalted on Stage-Tip columns according to Rappsilber
26 *et al.*²².
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38 Peptide fractions were loaded on to an Acclaim PepMap100, C₁₈, 100 Å, 75 μ m (internal
39 diameter) \times 15cm column using a Dionex UltiMate RSLCnano System (ThermoFisher
40 Scientific, UK). The peptides eluted by reversed-phase chromatography were analysed by a
41 microTOF-Q II mass spectrometer (Bruker Daltonics, Bremen, Germany) which was operated
42 in data-dependent mode, automatically switching between MS and MS/MS mode. The m/z
43 values of tryptic peptides were measured using a MS scan (300-2000 m/z), followed by MS/MS
44 scans of the six most intense peaks. Rolling collision energy for fragmentation was selected
45 based on the precursor ion mass and a dynamic exclusion was applied for 60 seconds.
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55 Raw spectral data were processed by DataAnalysis software (Bruker Daltonics, Bremen,
56 Germany) and the resulting peaklists were searched using Mascot 2.4 server (Matrix Science,
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3 UK) against the Uniprot Human sequence database containing 89,796 entries. Mass tolerance
4 on peptide precursor ions was fixed at 25ppm and on fragment ions at 0.06Da. The peptide
5 charge states were set as 2+ and 3+. iTRAQ modified N-terminal residue, iTRAQ modified
6 lysine and methylthio modified cysteine were set as fixed modifications, while oxidation of
7 methionine and iTRAQ modified tyrosine were set as variable modifications. The peptides
8 were filtered to a FDR of lower than 1%, estimated using the target-decoy search strategy.
9 iTRAQ quantification was performed by using WARPLC plugin on ProteinScape 3.1 software
10 (Bruker Daltonics, Bremen, Germany) to extract reporter ion intensities of every MS/MS
11 spectra with a requirement of two or more peptides. Peptide ratios were normalized based on
12 setting overall peptide median ratio at one, which corrects for unequal protein sampling, and a
13 coefficient of variability of peptide ratios were also determined for each quantified proteins.
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28 Arbitrary cut-off values of iTRAQ ratios of ≥ 1.2 and of ≤ 0.8 were applied to the data to
29 establish lists of differentially expressed proteins. Molecular functions and biological processes
30 were inferred using a variety of online tools including Reactome (reactome.org).
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38 **SDS-PAGE and Western blot analysis**

39 Approximately 50 μ g denatured protein samples were separated by 10-12% SDS-PAGE
40 before transfer to a nitrocellulose membrane. Membranes were blocked with 5% skimmed milk
41 protein before washing with PBST (PBS with 0.5% Tween-20). The membranes were then
42 probed with anti-myeloperoxidase [2C7] (#ab25989, Abcam, UK), anti-S100A9 [D5O6O]
43 (#72590, Cell signalling technology, USA) or anti- β -actin [D6A8] (# 8457, Cell signalling
44 technology, USA) primary antibodies at a dilution of 1:1,000 before detecting using the
45 relevant species HRP-conjugated secondary antibodies at a dilution of 1: 10,000. Bands were
46 detected using Novex ECL Chemiluminescent Substrate Reagent Kit (ThermoFisher
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3 Scientific, USA). Images were captured using GeneSnap software version 7.02 (SynGene,
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5 USA).

10 **Confocal microscopy**

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12 Neutrophils were seeded into a 6-well plate containing glass cover slips before infecting
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14 with *B. thailandensis* E555 at an MOI of 5 for 2 h as described above. Uninfected or *B.*
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16 *thailandensis* E555-infected neutrophils were fixed with 4% paraformaldehyde and
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18 permeabilized using 0.2% Triton-X 100 in PBS. Cells were then blocked with 3% BSA before
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20 incubating with anti-myeloperoxidase [2C7] (1:200; #ab25989, Abcam, UK), or anti- *B.*
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22 *thailandensis* E555 (1:100; kindly provided by Dr. Ganjana Lertmemongkolchai, Khon Kaen
23
24 University, Thailand). Cells were then incubated with goat anti-mouse⁵⁶⁸ (1:500; #A-11004,
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26 ThermoFisher Scientific, USA) and goat anti-rabbit⁴⁸⁸ (1:500; #A27034, ThermoFisher
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28 Scientific, USA) secondary antibodies and counter-stained with 4'-6-diamidino-2-phenylindole
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30 (DAPI; 1:500; Molecular probes, USA). After incubation, cover slips were mounted and
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32 analysed by laser scanning confocal microscopy (LSM800, Carl Zeiss, Switzerland).
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40 **Cytokine measurement by ELISA**

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42 Supernatants from both uninfected and infected cells were collected and filtered through
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44 0.22µm filters to remove any cell debris or intact viable bacteria. The concentrations of human
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46 IL-1β, IL-6, IL-8, TNF-α, IL-10 and IL-17A were determined using commercially available
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48 ELISA kits, according to the manufacturer's instructions. Samples from three independent
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50 experiments (n=3 biological repeats), each with three intra-experiment technical replicates
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52 were assayed.
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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 5×10^5 neutrophils were collected after 2 and 24 h post-infection before re-suspending with annexin-binding buffer. Then, 5 μ L of annexin V⁴⁸⁸ and 1 μ L of 100 μ g/ml propidium iodine (PI) were added to each tube. After 15 min incubation, 400 μ L 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

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3 subjects compared to cells isolated from diabetic donors was detected (Figure 1). This indicated
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5 that the diabetic neutrophils displayed a lower level of phagocytosis, as has been previously
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7 described ^{21, 24}. Despite a reduced level of initial bacterial uptake (T0), the neutrophils from
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9 diabetic volunteers displayed significantly higher levels of bactericidal activity when compared
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11 to neutrophils from healthy volunteers at both 2 and 4 hours post-infection (Figure 1). Although
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13 by 6 hours post-infection, no significant difference in bactericidal activity between neutrophils
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15 from healthy or diabetic donors could be observed (Figure 1). These results are in accordance
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17 with those described by Chanchamroen *et al.* who demonstrated that whilst neutrophils from
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19 diabetic patients are less phagocytic, they show increased rates of bactericidal activity against
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21 *B. pseudomalei* over time ²¹.
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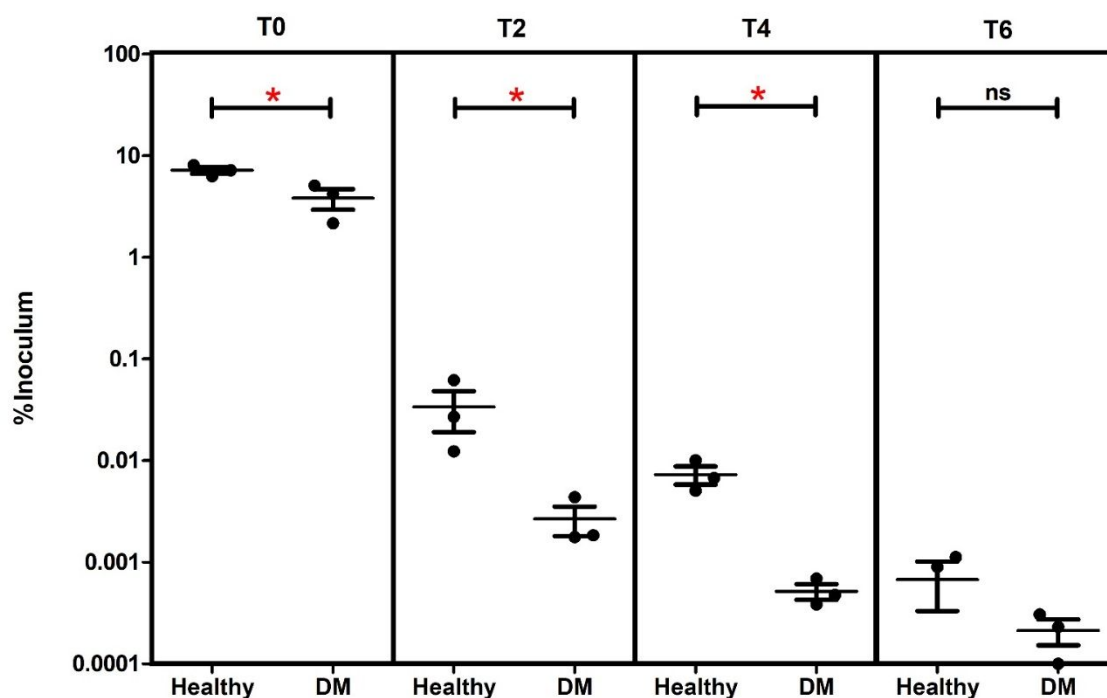


Figure 1. Comparison of the bactericidal activity of neutrophils isolated from healthy (NM) and diabetic (DM) volunteers. Neutrophils were infected with *B. thailandensis* E555 at an MOI of 5. Cells were lysed at 30 minutes (T0), 2 (T2), 4 (T4) and 6 (T6) hours post-infection for enumeration of viable bacteria. The number of intracellular live bacteria was expressed as a percentage of the initial inoculum using the equation [(number of intracellular viable bacteria at a single time point / number of bacteria in the inoculum)*100]. Each bar indicates the mean \pm 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

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2
3 ‘gluconeogenesis’ and ‘glucose metabolism’. Of significance when analysing the proteins with
4 increased expression in diabetic cells, were proteins mapping to the ‘antimicrobial peptides’
5 pathway. These proteins were Eosinophil cationic protein, Protein S100-A8, Protein S100-A9,
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‘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 pathogens²⁵. 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 neutrophils²⁶⁻²⁸. Resistin, originally identified as an insulin resistance factor²⁹ and more recently shown to be a potent activator of NETs³⁰, 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

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3 infected healthy neutrophils and 116 unique proteins were overexpressed in infected
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5 neutrophils from diabetics (Figure 2). The 30 proteins displaying the most extensive fold
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7 change in response to infection with this intracellular bacterium are listed in Table 1. This is
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9 the first proteomic comparison of the response of neutrophils from healthy and diabetic
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11 individuals. Mapping of these proteins onto molecular pathways using Reactome, confirmed
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13 that both infected healthy and diabetic neutrophils responded by increasing expression of
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15 proteins associated with the ‘innate immune system’ and ‘neutrophil degranulation’, with
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17 significantly more proteins mapping to these pathways from the infected diabetic cells. An
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19 increased number of proteins associated with innate immunity and neutrophil degranulation in
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21 infected diabetic neutrophils is suggestive of an increased inflammatory response by these cells
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23 compared to infected healthy neutrophils. Excessive neutrophil degranulation is a common
24
25 cause of many inflammatory diseases, such as severe asphyxic episodes of asthma, acute lung
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27 injury, rheumatoid arthritis, and septic shock ³¹. Further evidence for an increased
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29 inflammatory response of diabetic neutrophils upon infection, comes from the fact that a
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31 significant number of proteins from infected diabetic cells mapped to pathways involved in
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33 interferon and cytokine signalling, antigen presentation and antimicrobial peptides.
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35 Conversely, pathways mapping to the proteins increased in healthy infected cells were limited
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37 to those involved in ‘calcineurin activation of NFAT’, glycolysis and gluconeogenesis, ‘IL12
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39 and JAK-STAT signalling’.

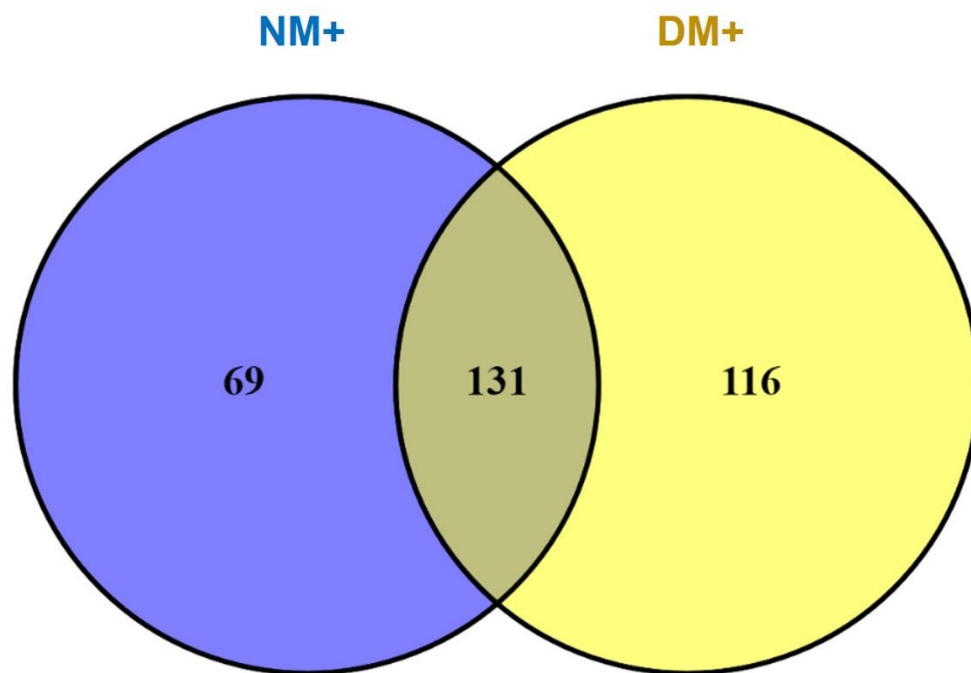


Figure 2. Venn diagram illustrating the numbers of common and unique proteins with increased expression upon infection of neutrophils isolated from either healthy or diabetic volunteers.

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).

Overexpressed in infected NM neutrophils			Overexpressed in infected DM neutrophils		
Accession no.	Protein description	Fold change (DM+/NM+)	Accession no.	Protein description	Fold change (DM+/NM+)
P60896	26S proteasome complex subunit SEM1	0.21	P12724	Eosinophil cationic protein	14.33
D6RC06	Histidine triad nucleotide-binding protein 1	0.22	K7EMV3	Histone H3	11.1
I3NI09	C-type lectin domain family 10 member A	0.27	Q9Y2K7-3	Isoform 3 of Lysine-specific demethylase 2A	10.45
Q8NA57	Uncharacterized protein C12orf50	0.29	P05164-2	Isoform H14 of Myeloperoxidase	8.63
P04406	Glyceraldehyde-3-phosphate dehydrogenase	0.3	A0A087WUA0	Fibrinogen alpha chain	6.97
P00558	Phosphoglycerate kinase 1	0.37	P11678	Eosinophil peroxidase	6.71
Q05315	Galectin-10	0.38	Q04760-2	Isoform 2 of Lactoylglutathione lyase	5.17
R4GN98	Protein S100	0.39	P08311	Cathepsin G	5.1
E5RIW3	Tubulin-specific chaperone A	0.4	O60814	Histone H2B type 1-K	4.51
P0DP23	Calmodulin-1	0.45	Q10567-4	Isoform 4 of AP-1 complex subunit beta-1	4.3
E7EQR4	Ezrin	0.46	E7ESK7	14-3-3 protein zeta/delta	4.04
H7C311	Hsc70-interacting protein	0.46	E9PMD7	Serine/threonine-protein phosphatase	3.64
P62328	Thymosin beta-4	0.47	E7EU81	Golgin subfamily B member 1	3.59
P38646	Stress-70 protein, mitochondrial	0.48	H7C320	Aldose 1-epimerase	3.56

P04083	Annexin A1	0.5	Q5W0S5	UV excision repair protein RAD23 homolog B	3.55
P05204	Non-histone chromosomal protein HMG-17	0.51	P48637	Glutathione synthetase	3.43
Q01518	Adenylyl cyclase-associated protein 1	0.52	E7ER44	Lactotransferrin	3.14
P62942	Peptidyl-prolyl cis-trans isomerase FKBP1A	0.53	P62805	Histone H4	3.01
H3BPC4	SUMO-conjugating enzyme UBC9	0.53	P15289-2	Isoform 2 of Arylsulfatase A	2.92
P07737	Profilin-1	0.54	P20618	Proteasome subunit beta type-1	2.74
P69905	Hemoglobin subunit alpha	0.54	I6L8B7	Fatty acid-binding protein, epidermal	2.65
P23284	Peptidyl-prolyl cis-trans isomerase B	0.54	F5H1S8	Malectin	2.59
C9JMM0	Chromobox protein homolog 3	0.55	P61626	Lysozyme C	2.53
Q32MZ4-3	Isoform 3 of Leucine-rich repeat flightless-interacting protein 1	0.55	K7ESA3	Synaptic vesicle membrane protein VAT-1 homolog	2.52
P14780	Matrix metalloproteinase-9	0.56	P80511	Protein S100-A12	2.49
B7ZBK6	Delta-aminolevulinic acid dehydratase	0.56	C9J1Z8	ADP-ribosylation factor 5	2.47
M0QYG6	Uncharacterized protein	0.56	H7BZJ3	Protein disulfide-isomerase A3	2.37
P68036-2	Isoform 2 of Ubiquitin-conjugating enzyme E2 L3	0.58	P07384	Calpain-1 catalytic subunit	2.32
P52566	Rho GDP-dissociation inhibitor 2	0.59	A0A0C4DFS8	Nicotinamide phosphoribosyltransferase	2.27
Q92820	Gamma-glutamyl hydrolase	0.61	P05109	Protein S100-A8	2.26

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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.

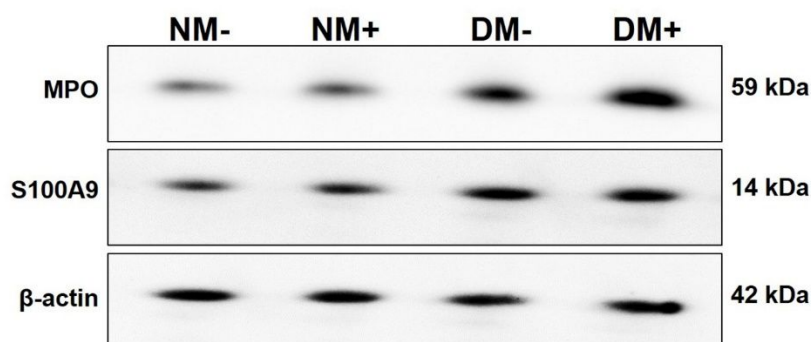


Figure 3. Western blot analysis of Myeloperoxidase and S100-A9 in uninfected and *B. thailandensis* E555-infected healthy and diabetic neutrophils. Cells were incubated with media (uninfected) or with *B. thailandensis* E555 at an MOI of 5 for 2 hours. After lysis, separation by SDS-PAGE and transfer 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.

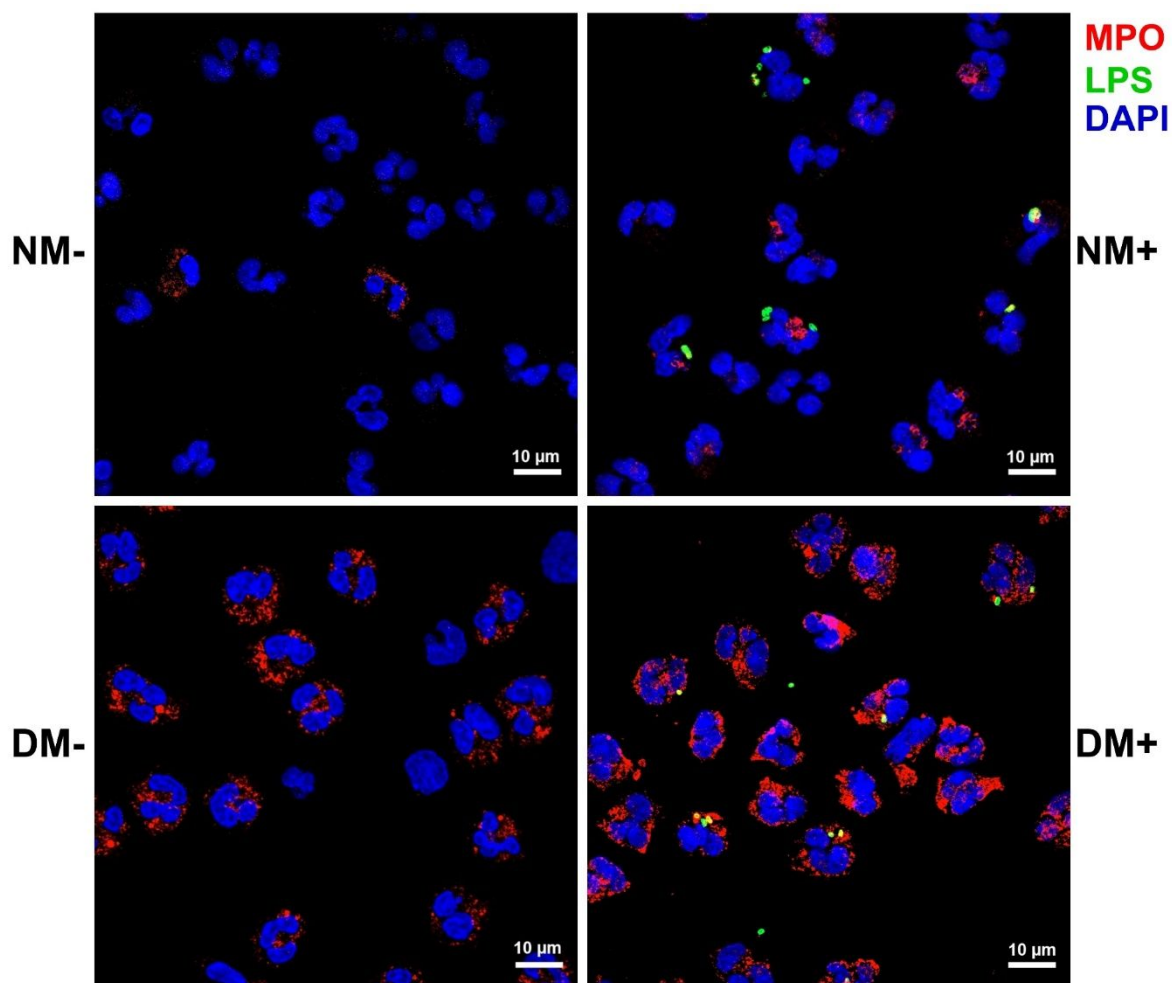


Figure 4. Cellular localisation of Myeloperoxidase in uninfected and *B. thailandensis* E555-infected healthy or diabetic neutrophils. Neutrophils were incubated in media or with *B. thailandensis* E555 at an MOI of 5 for 2 hours. Cells were fixed and permeabilized before immunostaining with mouse anti-MPO and rabbit anti-LPS primary antibodies. Cells were then probed with goat-anti mouse⁵⁶⁸ and goat anti-rabbit⁴⁸⁸ 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 = 10 μ m.

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3 Myeloperoxidase and calprotectin are also molecules extruded by neutrophils in NETs
4 upon infection. We detected 18 NET-associated proteins ²⁶⁻²⁸ that were overexpressed in
5 diabetic neutrophils compared to healthy neutrophils in response to infection (Table 2),
6 although we did not investigate NET formation in this study. Whilst our data conflicts with a
7 previous report that suggested that neutrophils from diabetic patients were less able to produce
8 NETs in response to infection with *B. pseudomallei* ¹⁵, it is in agreement with a second study
9 illustrating that NET markers are significantly elevated in the plasma of DM patients compared
10 to healthy controls, and that these markers are further increased in DM patients with
11 melioidosis ³⁴. However, an alternative hypothesis could be that the biological difference in
12 neutrophil responses lies in the ability of the cells to undergo degranulation, since many of the
13 proteins in Table 2 and within the overall list of 116 proteins overexpressed by infected
14 neutrophils from diabetic donors are associated with neutrophil degranulation and are potent
15 inflammatory mediators.
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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.

Accession no.	NET-associated protein	Fold change
P12724	Eosinophil cationic protein	14.3
K7EMV3	Histone H3	11.1
P05164-2	Isoform H14 of Myeloperoxidase	8.6
P08311	Cathepsin G	5.1
O60814	Histone H2B type 1-K	4.5
E7ER44	Lactotransferrin	3.1
P62805	Histone H4	3.0
P61626	Lysozyme C	2.5
P80511	Protein S100-A12	2.5
P05109	Protein S100-A8	2.3
C9JVG0	Serotransferrin	2.1
Q9HD89	Resistin	1.8
P06702	Protein S100-A9	1.7
P35579	Myosin-9	1.4
P12814	Alpha-actinin-1	1.3
P59665	Neutrophil defensin 1	1.3
U3KPS2	Myeloblastin	1.3
P25774	Cathepsin S	1.3

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 TNF α , IL-6, IL-8 and IL-12p70 in whole blood of diabetic subjects compared to healthy subjects³². We assessed the levels of TNF α , IL-6, IL-1 β 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, TNF α , IL-6 and IL-1 β were elevated in the supernatants of both diabetic and healthy infected neutrophils (Figure 5A). Furthermore, TNF α , IL-6 and IL-1 β 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-1 β 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

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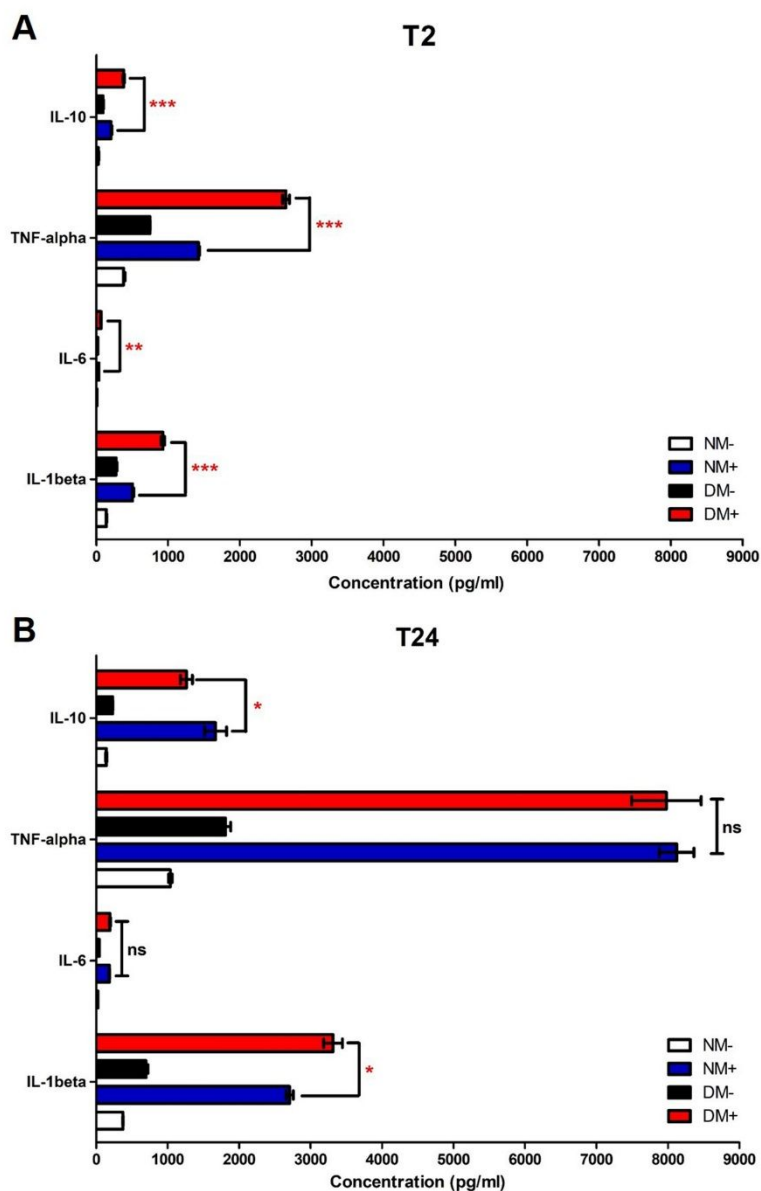


Figure 5. Cytokine secretion by uninfected and E555-infected neutrophils from healthy and diabetic subjects. Neutrophils from healthy (NM) or diabetic (DM) volunteers were incubated with media or infected with *B. thailandensis* E555 at an MOI of 5 for 2 hours (T2) (A) and 24 hours (T24) (B). Secretion of IL-10, TNF α , IL-6 and IL-1 β 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 \pm SEM for data obtained from three independent experiments (n=3 biological replicates), where * = p-value \leq 0.05, ** = p-value \leq 0.01 and *** = p-value \leq 0.001.

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3 Neutrophils are a significant source of the pro-inflammatory cytokine IL-17, a cytokine
4 with autocrine function which increases expression of other pro-inflammatory cytokines,
5 proteases and reactive oxygen species, upon LPS stimulation ³⁵. IL-17 has been linked to
6 effective anti-fungal responses but also to chronic inflammatory conditions such as rheumatoid
7 arthritis and psoriasis, and has been shown to be elevated in the circulation of Type 2 diabetes
8 patients ³⁶. Here we studied the secretion of IL-17A in neutrophils from healthy and diabetic
9 subjects treated with media or BTCV E555 for 2 and 24 hours. Cells from healthy volunteers
10 failed to stimulate an appreciable level of secreted IL-17, regardless of infection status (Figure
11 6). Conversely, uninfected neutrophils from diabetic volunteers secreted readily detectable
12 levels of IL-17 in the absence of infection (between 50-60pg/ml at both 2 and 24 hours) (Figure
13 6). Significantly, levels of secreted IL-17 increased further upon infection of the diabetic
14 neutrophils, and over time.
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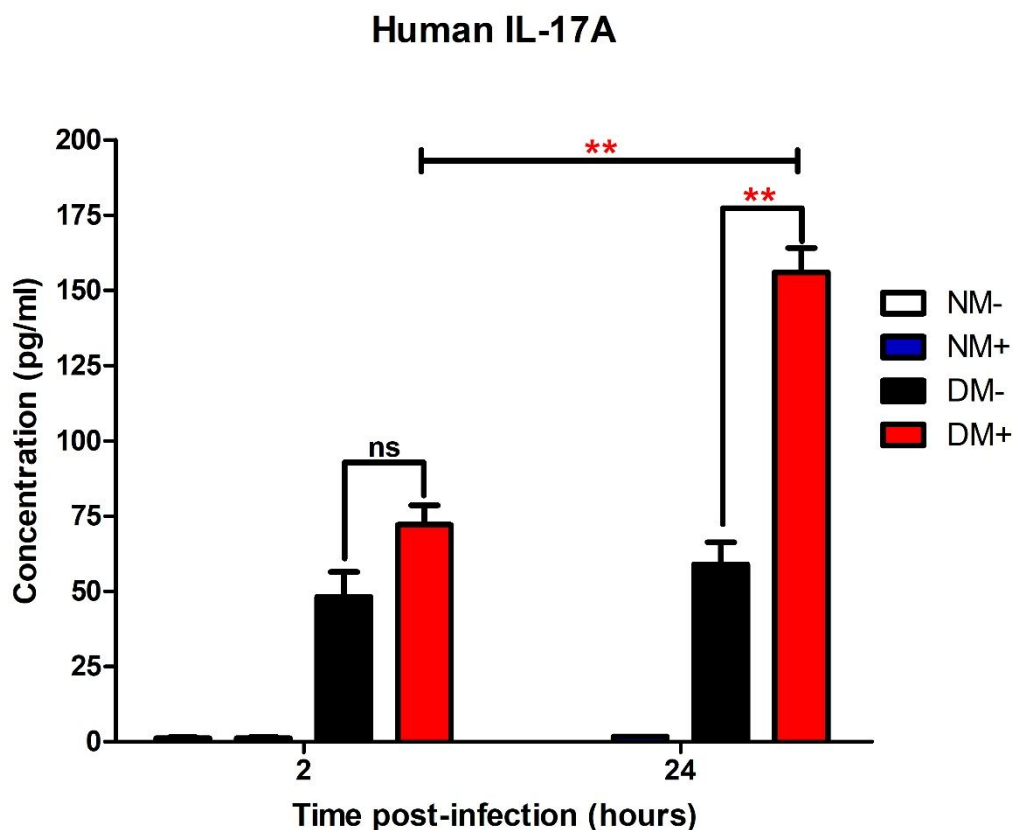
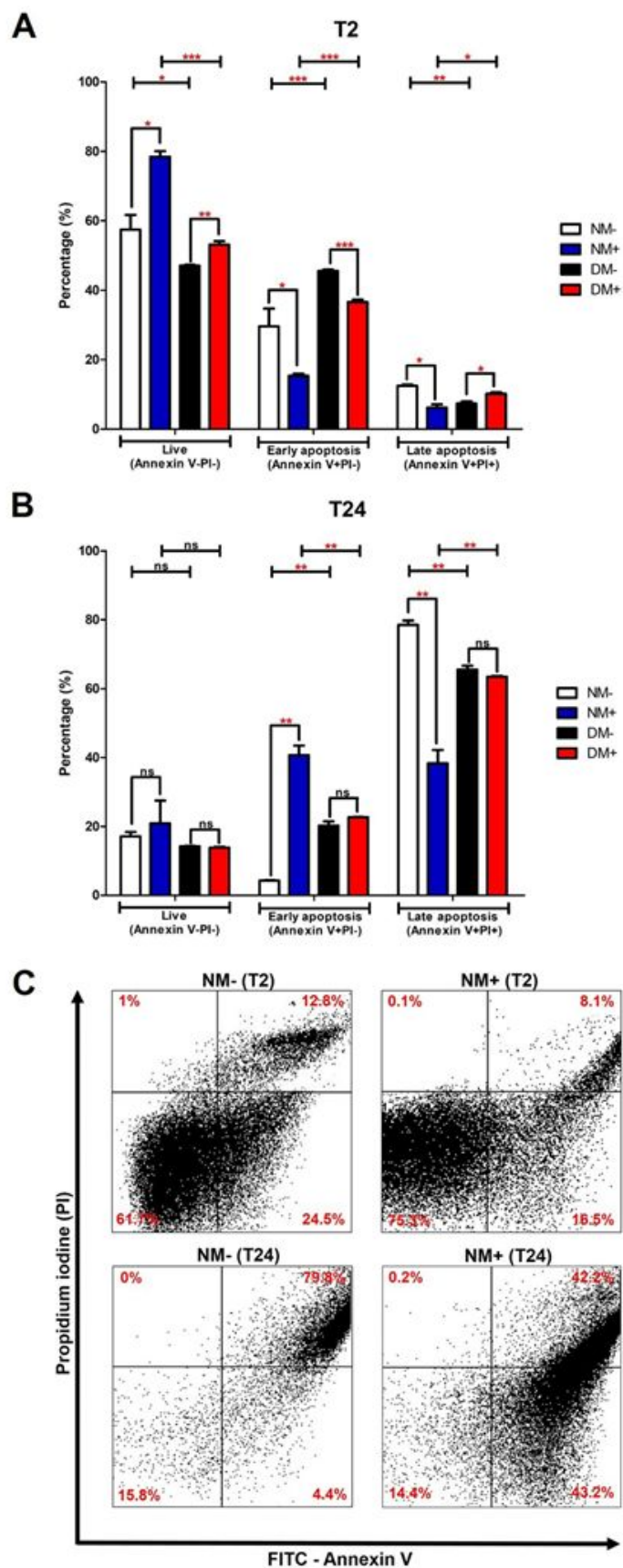


Figure 6. Secretion of IL-17 by uninfected and E555-infected neutrophils from healthy and diabetic subjects. Neutrophils from healthy (NM) or diabetic (DM) volunteers were incubated with media or infected with *B. thailandensis* E555 at an MOI of 5 for 2 hours (T2) (A) and 24 hours (T24) (B). Secretion of IL-17 in cell supernatants was detected by ELISA. Each bar indicates the mean \pm 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 α , 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 V⁴⁸⁸ 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

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3 possibly undergoing cell death through necrosis or an alternative form of cell death involving
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5 membrane disintegration.
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3 **Figure 7. Assessment of apoptosis of uninfected and *B. thailandensis* E555-infected**
4 **neutrophils from healthy or diabetic individuals.** Neutrophils were treated with media or
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6 infected with *B. thailandensis* E555 at an MOI of 5 for 2 hours (T2, A) and 24 hours (T24, B).
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8 Cells were stained with annexin V⁴⁸⁸ and propidium iodine (PI) before flow cytometry analysis.
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10 Each bar indicates the mean \pm SEM for data obtained from three independent experiments (n=3
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12 biological replicates). An unpaired Students *t*-test was used to compare the means of two
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14 independent sample groups, where * = p-value \leq 0.05, ** = p-value \leq 0.01 and *** = p-value
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16 \leq 0.001. Representative plots of data obtained from NM neutrophils before and after infection
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18 at 2 (T2) and 24 (T24) hours post-infection are shown in C.
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29 One classic feature of cell death by necrosis that distinguishes this process from
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31 apoptosis, is a loss of plasma and nuclear membrane integrity. This can be measured by
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33 assessing the levels of Lactate dehydrogenase in cell supernatants, a cytoplasmic enzyme which
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35 is only present in the extracellular media upon disintegration of the plasma membrane. Infected
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37 healthy neutrophils display decreased levels of cell lysis compared to the uninfected control
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39 cells, at both 2 and 24 hours post-infection (Figure 8). Conversely, infection of neutrophils
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41 isolated from diabetic donors displayed a consistent and significant increase in cell lysis at both
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43 2 and 24 hours post-infection (Figure 8). This data suggests that neutrophils from diabetic
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45 subjects are more likely than neutrophils from healthy donors to undergo a form of lytic cell
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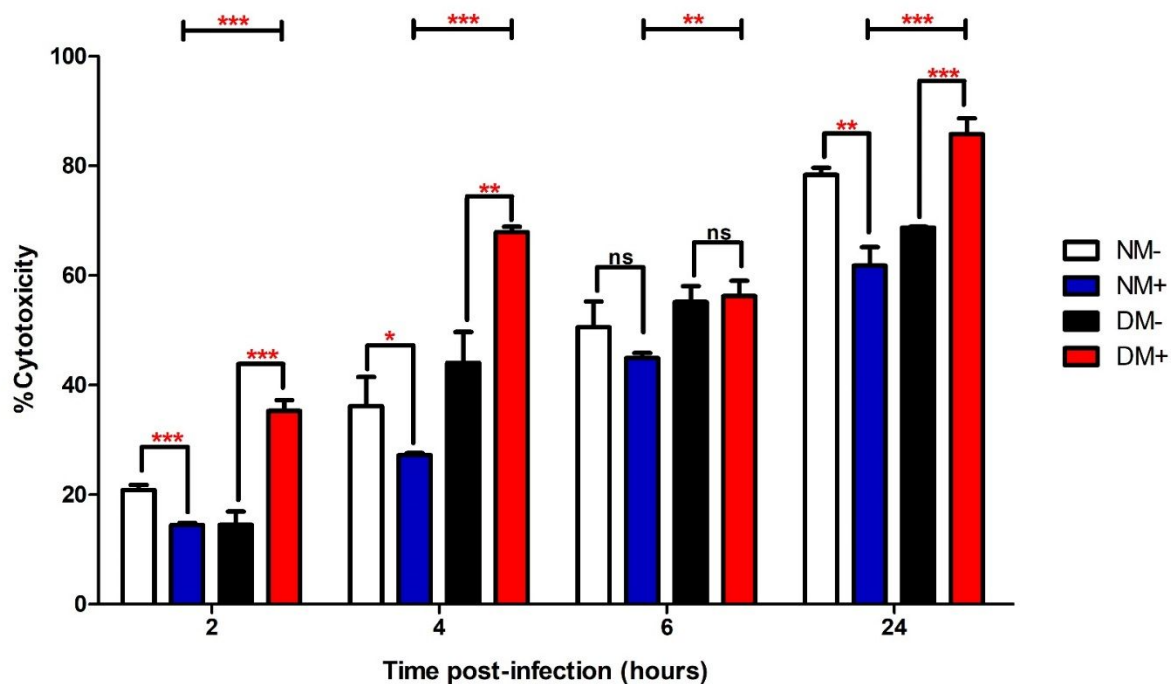


Figure 8. Assessment of cell integrity of uninfected and *B. thailandensis* E555-infected neutrophils from healthy and diabetic individuals. Neutrophils were treated with media or infected with *B. thailandensis* E555 at an MOI of 5 for 2 hours (T2) and 24 hours (T24). Cell supernatants were harvested and assayed for extracellular lactate dehydrogenase (LDH). Each bar indicates the mean \pm 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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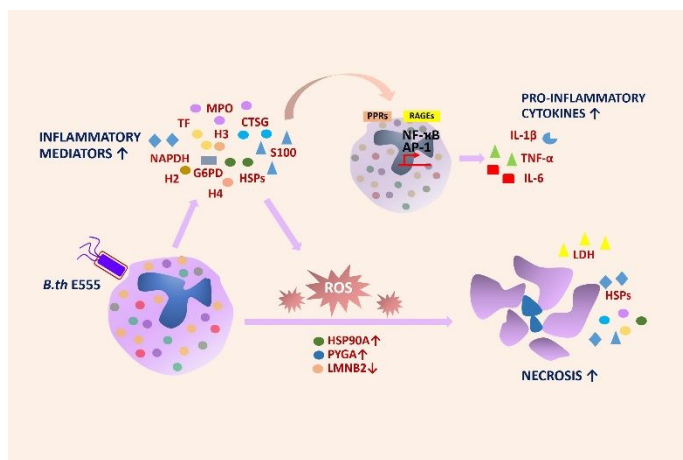
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For TOC Only



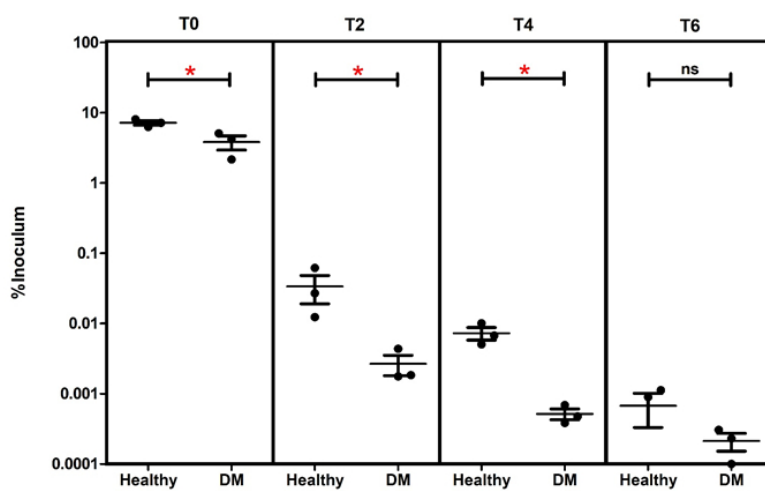


Figure 1

190x275mm (96 x 96 DPI)

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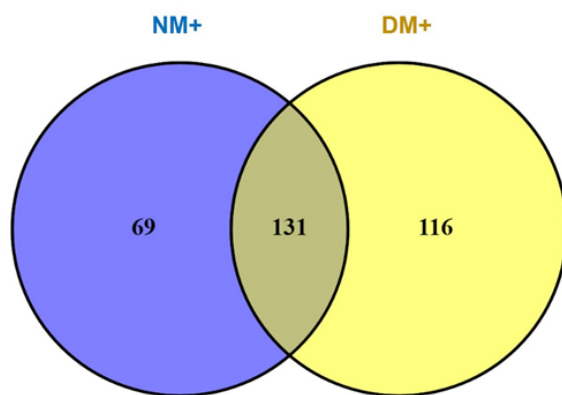


Figure 2

190x275mm (96 x 96 DPI)

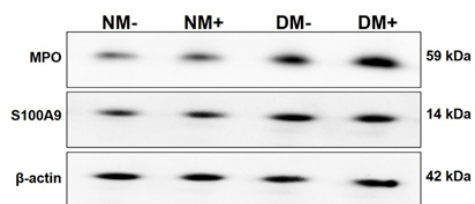


Figure 3

190x275mm (96 x 96 DPI)

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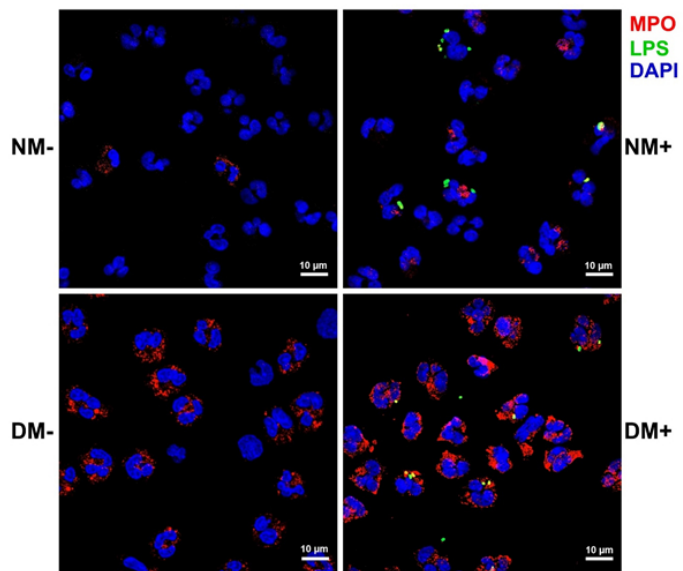


Figure 4

190x275mm (96 x 96 DPI)

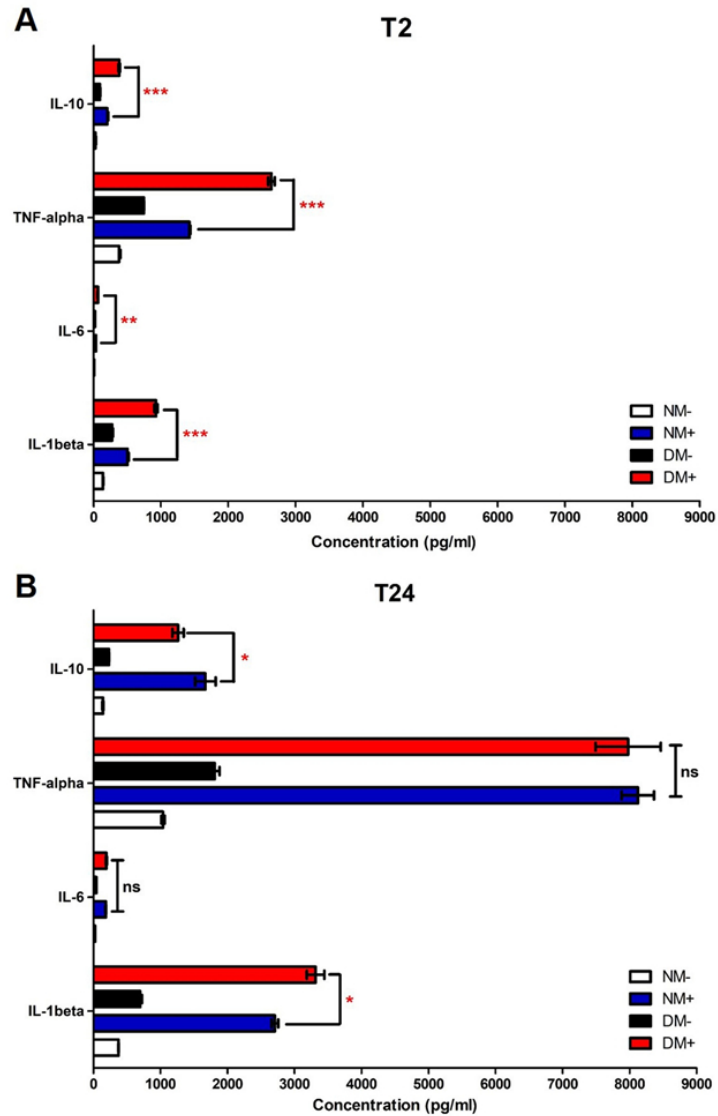


Figure 5

190x275mm (96 x 96 DPI)

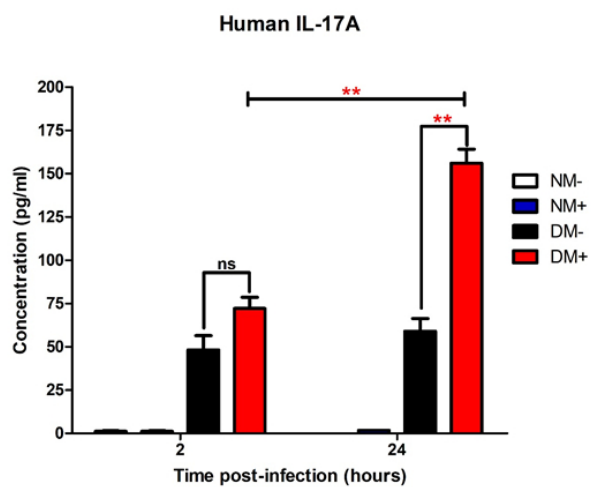


Figure 6

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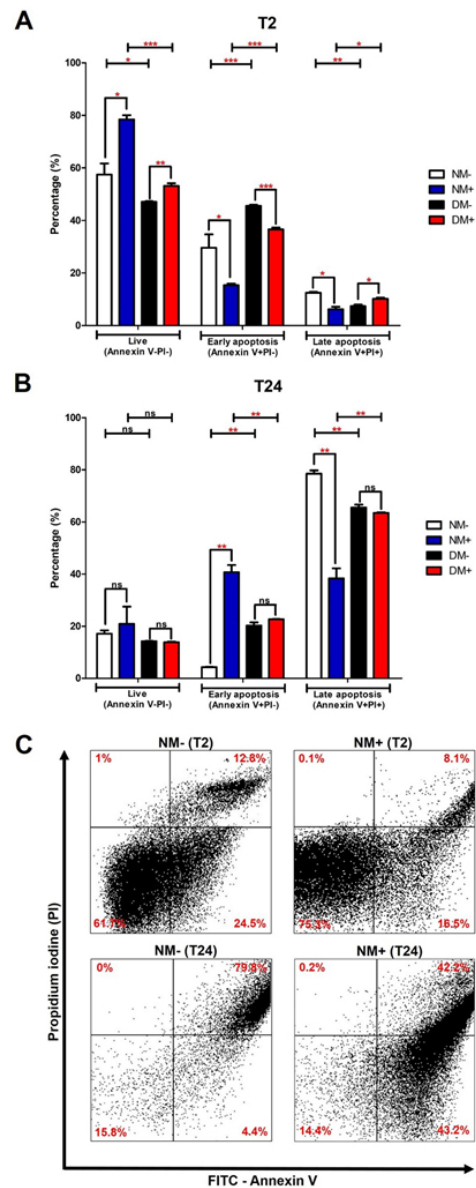


Figure 7

190x275mm (96 x 96 DPI)

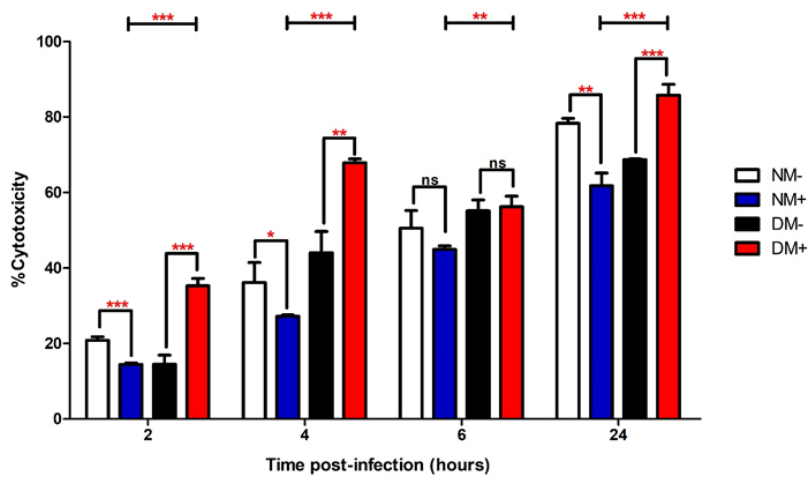


Figure 8

190x275mm (96 x 96 DPI)