Gene expression profile of human cytokines in response to *B.pseudomallei* infection

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ABSTRACT

Melioidosis, is an under-reported infectious disease, caused by the gram-negative bacterium *Burkholderia pseudomallei.* Understanding disease pathogenesis and susceptibility is crucial for developing newer diagnostic and therapeutic strategies for this life threatening infection. In this study, we aimed to analyze the gene expression levels of important cytokines in melioidosis patients and establish useful correlates with disease biomarkers compared to cases of sepsis infection caused by other pathogens and healthy individuals. A Qiagen common human cytokines array, profiling the gene expression of 84 important cytokines by real time quantitative polymerase chain reaction (RT qPCR) was used. We analyzed 26 melioidosis cases, 5 healthy controls and 10 cases of sepsis infection caused by other pathogens. Our results showed a consistent up regulated expression of interleukins; IL4, IL17A, IL23A, IL24, interferons; IFNA1, IFNB1, Tumor necrosis factor (TNF) super family; TNFSF4, Transforming growth factor (TGF) superfamily; bone morphogenetic protein 3,6 (BMP3, BMP6), TGFB1,other growth factors; macrophage colony stimulating factor (M-CSF) , C-fos induced growth factor (FIGF) and platelet derived growth factor alpha polypeptide (PDGFA) in melioidosis patients compared to other sepsis cases, irrespective of comorbidities, duration of fever/clinical symptoms and antibiotic treatment. Our findings indicate a dominant Th2 and Th17 type cytokine responses, suggesting that their dysregulation at initial stages of infection may play an important role in disease pathogenesis.IL1A, IL1B and IL8 were significantly down regulated in septicaemic melioidosis patients compared to other sepsis cases. These differentially expressed genes may serve as biomarkers for melioidosis diagnosis, as targets for therapeutic intervention and help us understand immune response mechanisms.

IMPORTANCE

Melioidosis is a life threatening infectious disease caused by a soil-associated gram-negative bacterium, *B. pseudomallei*. Melioidosis is endemic in Southeast Asia and northern Australia; however, the global distribution of *B.pseudomallei* and the disease burden of melioidosisis still poorly understood. Melioidosis is difficult to treat as *B.pseudomallei* is intrinsically resistant to many antibiotics and requires a long course of antibiotic treatment. Mortality rate remains high in endemic areas with reoccurrence being common. Therefore, it is imperative to diagnose the disease at an early stage and provide vital clinical care to reduce the mortality rate. With limitations in treatment and lack of a vaccine, it is crucial to study the immune response mechanisms to this infection to get a better understanding of disease pathogenesis and susceptibility. Therefore, this study aimed to analyze the gene expression levels of important cytokines to establish useful correlation for diagnostic and therapeutic purposes.

INTRODUCTION

Melioidosis is a life threatening infectious disease and is endemic in Southeast Asia and northern Australia (1). A recent report estimates melioidosis disease burden to be 165,000 cases per year (2). Lack of awareness of melioidosis disease among physicians and lack of diagnostic methods contribute to underreporting in many endemic countries. Infection is suspected to be acquired mainly via skin during exposure to soil and contaminated water. Nevertheless inhalation of aerosolized bacteria during extreme weather events such as rainfall and storms has also been reported (2, 3).The disease is strongly associated with comorbidities such as diabetes mellitus, chronic kidney disease, thalassemia, immunosuppression and excessive

alcohol intake (1, 4, 5). A broad spectrum of clinical presentations ranging from acute fulminant septicemia to chronic localized abscesses are reported for melioidosis (5). Early diagnosis and appropriate antibiotic treatment plays a crucial role in preventing mortality and recurrence. Advancement of new immunodiagnostic methods and therapeutic strategies is important for disease management of melioidosis, given the lack of vaccines and limitations in drug treatment (3).

Studying the host immune responses to infection is crucial for understanding disease pathogenesis, susceptibility and immune correlates of protection (3). Cytokines are vital immune modulators that regulate and determine the nature of immune responses to an infection (6). Activation of leukocytes and cytokine networks are prominent features of inflammation and the septic response (7). Pro- and anti-inflammatory cytokines play a critical role in regulating overall immune responses and in establishing homeostasis, and their dysregulation is instrumental in triggering disease progression and severity (8). Hence a detailed study of the cytokine cascade events at the transcriptome level during an infection is useful to understand disease pathogenesis and susceptibility. Although cytokine cascade events following *B.pseudomallei* infection have been studied in several animal models (7, 9-13), data on human host mRNA expression levels of cytokines is limited. Pro-inflammatory cytokines such as IL8, IL6, IL12, IL18, IL15, IFNγ, TNFα, IL1β, anti-inflammatory cytokines such as IL4 and several other chemokines have been implicated in disease outcome during the early acute phase of *B.pseudomallei* infection (7, 14-16).While individual cytokines have been investigated in previous studies, the profiling of entire cytokine networks is necessary to comprehensively understand specific immune response pathways and thereby the pathophysiology of melioidosis. Such a profile may also help identify disease biomarkers with therapeutic implications.

We have successfully established a nation-wide surveillance system in Sri Lanka which has resulted in finding more confirmed cases of melioidosis (unpublished data). In this study we aimed to analyze the gene expression profiles of important human cytokines in Sri Lankan melioidosis patients to further understand the immune response mechanisms during melioidosis and establish useful correlates with disease biomarkers.

METHODS

Patient enrollment

Nationwide active surveillance for melioidosis was established in multiple state and private hospitals throughout Sri Lanka, with ethics approval from the Ethics Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka and the Office of Human Research Protection (OHRP), United States Army Medical Research and Material Command (USAMRMC). Patients fitting the clinical case definition of melioidosis i.e. febrile illness for more than 5 days, pneumonia, septic arthritis, skin lesions, septicaemia, lung, soft tissue or deep abscess were recruited for initial screening for melioidosis. Blood, pus and other patient specimens were collected for bacterial cultures and serum samples were collected for indirect haemagglutination (IHA) antibody test. Any positive bacterial cultures were further screened and confirmed as *B.pseudomallei* by PCR. All samples for the study were collected between September 2014 and April 2016.

Patients who were culture positive for *B. pseudomallei* and / or had high antibody titers (>640) by the IHA test were recruited for our study and classified as positive cases of melioidosis. Culture and PCR positive samples were considered as confirmed cases of melioidosis. Samples with an antibody titre of >640 by IHA testing were

considered as probable cases of melioidosis. At the time of recruitment all melioidosis patients were undergoing antibacterial treatment.

We also recruited healthy donors and patients fitting the clinical definition of severe sepsis/septic shock (as per the 2012 WHO guidelines for sepsis management) who were negative for *B. pseudomallei*, as negative controls for our gene expression profiling study (17).

Bacterial culture and identification

Primary isolation of *B. pseudomallei* was done at the admitting hospital using conventional culture techniques for blood, sputum, pus and other specimens. Bacterial isolates that were oxidase positive, gentamicin-resistant and gram-negative bacilli were forwarded to the reference laboratory in Colombo where they were sub-cultured to establish pure growth and maintained at −70°C in 15% brain heart infusion (BHI) glycerol for subsequent definitive tests. Bacteria were resuscitated by subculture onto 5% blood agar and incubated for 24 h at 37 °C to give single colony growth for all subsequent tests.

Real time PCR assay for confirmation of *B.pseudomallei*

A single colony of *B. pseudomallei* grown on blood agar from patients sample was re-suspended in ultrapure water. The suspension was heated at 95 °C for 10 min and centrifuged at 13500 x g to pellet the cell debris. The supernatant was used as the template for all subsequent PCR assays. Real time PCR assay was done for gene targets of the lpxO, YLF and BTFC gene clusters using the primers and methods described previously (18, 19).

IHA antibody testing

Antibody testing against *B.pseudomallei* antigen was performed using an in-house method adapted from Alexander et al, 1970 (20). Antigen was prepared from heat killed culture supernatant of a Sri Lankan *B.pseudomallei*, strain BPs7. A 1/80 diluted antigen preparation was used to sensitize sheep erythrocytes. Serum samples were heat inactivated at 56°C for 30 mins and tested by serial dilution from 1/10 to 1/10,240 with sensitized sheep erythrocytes and the reciprocal of the highest dilution at which hemagglutination occurred was recorded as end point titer (20).

Sample collection and processing

10 ml of whole blood was collected from patients/volunteers after written informed consent, of which 7ml were collected into B.D vacutainer mononuclear cell preparation tubes (catalog 362761) for lymphocyte purification. The lymphocytes were purified using the Ficoll fractionation method as per manufacturer's instructions and lysed with RLT buffer (Qiagen RNeasy mini kit-catalog 74104), homogenized and stored at -80°C for total RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the stored cell lysate samples using the Qiagen RNeasy mini kit (catalog no:74104) as per manufacturer protocol. RNA extracted from 0.6 million PBMC's was used for cDNA synthesis as the standard for all samples analyzed by RT-qPCR. cDNA was synthesized using Qiagen First strand kit (catlog330401) as per the manufacturer recommended instructions. The synthesized cDNA samples were stored at -20°C until further use.

Real Time qPCR and gene expression analysis

Qiagen human common cytokines RT^2 Profiler PCR array (catlog PAHS-021Z) was used for this study. The PCR reaction and thermal profile recommended by the manufacturer were followed. 26 melioidosis cases (identified as confirmed or probable cases), 10 other bacterial sepsis cases (negative for *B.pseudomallei*) and 5 healthy negative controls were analyzed by RT-qPCR.

Data Analysis

The relative gene expression ratio, for measuring the change in expression level of a gene was calculated by delta delta CT method (21) as per manufacturer recommendations. The data was normalized using actin beta as the reference housekeeping gene. Statistical analysis was done by Welch's T-test using SAS PROC MIXED, version 9.4. $P<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

A total of 26 cases of melioidosis were analyzed of which 23 were confirmed cases (culture positive), and 3 were probable cases (high antibody titre positive). A majority (n=23) of melioidosis cases had associated comorbidities, and diabetes was the most common comorbidity (n=17) in this study. Out of 23 confirmed cases of melioidosis 16 were classified as septicaemic or bacteriaemic.

The differential expression pattern of interleukins (IL), interferons (IFN), tumor necrosis factor (TNF) super family, transforming growth factor (TGF) super family and other growth factors was significant in melioidosis patients compared to other bacterial sepsis infection cases and healthy controls. Adiponectin, C1Q and collagen domain containing (ADIPOQ) and family with sequence similarity 3, member B

(FAM3B) were significantly down regulated in other bacterial sepsis infection cases compared to healthy controls (fig 1).

Gene expression profile of interleukins

Our study reveals up regulated expression of IL10, IL1B, IL1RN (interleukin 1 receptor antagonist), IL27 and IL8 in melioidosis patients compared to healthy controls (Table 1, Fig 1).This is in agreement with a study by Weirsinga et al, 2007 reporting increased mRNA expression of inflammatory response genes such as IL1β, IL6, IL15, IL10, IL4, IFNγ and TNFα in melioidosis patients when compared to healthy controls (7). In our study, IL16, IL17A, IL23A and IL24 were down regulated while IL10, IL1B and IL8 were up regulated in other bacterial sepsis infection cases compared to healthy controls (Fig 1). IL16, IL17A, IL17B, IL1RN, IL22, IL23A, IL24, IL27, IL3 and IL4 were all up regulated in melioidosis patients compared to other bacterial sepsis infection cases (Table 2, Fig 1). Particularly, IL17A, IL3 and IL4 showed high levels of gene expression. Previously, expression profiling of interleukins in response to *B.pseudomallei* infection has been extensively studied in several animal models, showing upregulated expression of interleukins such as IL1β, IL6, IL10 and IL12 within 72 hours of infection (9-13, 15). Elevated levels of expression of IL6, IL8, IL12, IL15 and IL18 was also observed in the plasma of melioidosis patients (15, 16).

IL17A, a pro-inflammatory cytokine which mediates inflammatory responses and induces production of other cytokines, is particularly expressed at very high levels in melioidosis patients (including septicaemic and diabetic cohorts) compared to other sepsis infections (Fig 1- 2). Additionally, IL-22 a widely regarded Th17 cytokine, also shows upregulated expression in melioidosis patients compared to other sepsis cases.

IL17 and other Th17 cytokines are linked to the response against extra-cellular bacteria, pathogenesis of diverse autoimmune and inflammatory diseases, as their dysregulated expression can lead to uncontrolled inflammatory responses (22, 23). IL-17 is also implicated in excessive tissue damage by stimulating the production of many other cytokines including granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), TGF-β, TNF-α, thus contributing to inflammatory pathology (8). IL23, a key mediator of inflammation has also been reported to show upregulated mRNA expression during *B.pseudomallei* infection, implicating its role in pathogenic host immune responses (24). Anti-IL17 and anti-IL23 therapeutic agents have shown to be effective in several immune-mediated inflammatory diseases (23, 25). IL27, implicated in regulating B and T cell activity, has been reported to be significantly elevated in melioidosis patients compared to healthy controls and over production of IL27 plays a major role in pathogenesis of sepsis and shock (26). IL27 has also been identified as a potential sepsis biomarker and a candidate in successful therapeutic intervention (27, 28).As our results show consistent upregulated expression of IL17, IL23 and IL27, their role in melioidosis disease progression and therapeutic use should be further investigated.

Our study revealed greater than 3 fold upregulation of IL4, IL13, IL17A, IL17B, IL22, IL23A, IL24 and IL27 in the diabetic melioidosis cohort (n=17) compared to other bacterial sepsis cases (Fig 2).Diabetes, a risk factor for infectious diseases, may play a role in neutrophil and T-cell dysfunction, possibly mediated by altered glucose metabolism and oxidative stress (29). Studies on diabetic cohorts (mice and human) of melioidosis infection shows excessive neutrophil infiltration and impaired inflammatory and Th1 cytokine responses, leading to increased susceptibility of diabetic individuals to melioidosis (10, 30). IL4, a key regulator of humoral and

adaptive immunity, functions as an anti-inflammatory cytokine decreasing production of Th1 cells and related pro-inflammatory cytokines. Our findings show upregulation of IL4 and closely related anti-inflammatory cytokine IL13, in the melioidosis cohort compared to other sepsis cases, which is suggestive of inflammatory responses being dysregulated. Upregulated IL4 expression has been reported in melioidosis patients and acute melioidosis animal models (7, 9).

Our findings also show significant upregulation of IL17A, IL17B, IL23A whereas IL1A, IL1B and IL8 were down regulated in septicaemic melioidosis cohort (n=16) compared to other sepsis cases (Fig 1). Downregulation of IL1A, IL1B, IL6, IL8 and IL21 in the early acute phase melioidosis cohort (<15 days fever/clinical symptoms) compared to other sepsis cases (Fig 3) was also seen, indicating IL1A, IL1B and IL8 as potential markers during the early stages of inflammation and being correlated with disease severity. A study using a human lung epithelial cell line showed that IL8 production upon *B.pseudomallei* infection was lower than cells infected with other gram negative bacteria which correlates with our findings (14). Increased level of plasma IL6 and IL8 concentration, being associated with disease severity and mortality have also been reported (15). Immunosuppression in melioidosis patients correlating to mortality, associated with up regulated interleukin-1R-associated-kinase-M expression, leading to a strong decrease in capacity to release pro-inflammatory cytokines such as IL1B, TNFα and IL8, after *ex-vivo* stimulation with LPS or *B.pseudomallei*, has been reported (31). Downregulation of IL1B upon *B.pseudomallei* infection compared with avirulent *B.thailandensis* in infected lung epithelial cells has also been reported, suggesting host response evasion (32). In our findings, we also see an upregulation of ILRN, a natural inhibitor of pro-inflammatory effects of IL1A, IL1B, in melioidosis patients compared to other

bacterial sepsis cases. Thus IL1 and IL8 which are key mediators of inflammation and early innate immune responses, may serve as candidate early diagnostic markers and indicators of disease severity.

Our findings reveal an upregulated Th2, Th17 cytokine response and a down regulated Th1 cytokine response, with associated comorbidities such as diabetes playing a key role in pathogenesis and severity through dysregulated cytokine responses.

Gene expression profile of interferons

Interferon A5 (IFNA5) was down regulated in melioidosis patients compared to healthy controls (Table 1, Fig 1). Interferon B1 (IFNB1) was significantly down regulated in other sepsis infection cases compared to healthy controls (Fig 1). Interferon A1(IFNA1) and IFNB1 showed upregulation whereas IFNA5 was down regulated in the melioidosis patients compared to other sepsis infection cases (Table 2, Fig 1).

Elevated expression of interferon gamma (IFNγ), a pro-inflammatory cytokine, has been reported in human host and animal models of *B. pseudomallei* infection during the early stages (7, 11, 12, 16). Our findings did not show any significant upregulation of INFγ in melioidosis cases compared to healthy controls or other bacterial sepsis cases, possibly due to our samples being collected at latter stages while undergoing antibiotic treatment. However we did see a significant upregulation in the diabetic melioidosis cohort compared to other sepsis cases of infection (Fig 2). Interferon mediated responses have been reported as the most dominant pathway, with class I and II interferons being prominent in melioidosis and tuberculosis infections (33). Our study shows an upregulated expression of INFA1 and INFB1 in melioidosis

patients (including diabetic and septicaemic cohorts) compared to other sepsis infections (Fig 1,2). Interferons α and β, both belonging to class I interferons, play a major role in innate immune responses. Dysregulated type I IFN production results in a damaging cascade of cell death, inflammation, and immunological host responses that can lead to tissue injury and disease progression (34). Studies have shown type I IFN responses as a striking characteristic of TB infection and that lack of development of Th1 immunity in response to *M. tuberculosis* appears to be associated with increased induction of type 1 IFNs, leading to better bacterial survival and host evasion (35). Furthermore a study also reported that type 1 IFNs suppresses IL-1 production, providing cellular basis for the anti-inflammatory effects, as well as pro-bacterial functions of type I IFNs during *M. tuberculosis* infection (36). Our findings show a similar response, as we see a dominant type I IFN production and a fairly submissive IFNγ and related Th1 cytokines production in melioidosis patients. This data supports our findings of significant downregulated expression of IL1A, IL1B in melioidosis patients compared to other sepsis cases. Type I interferons are also considered as mediators of endotoxic shock and sepsis induced by gram negative bacteria, with IFNB and IFNAR1 deliberated as therapeutic targets (37, 38). Thus further investigation is required to understand the expression of class I interferons in relation to pathogenesis of melioidosis and its role in diagnostic and therapeutic intervention.

Gene expression profile of TNF superfamily

Our findings reveal an upregulation of TNFα an important pro-inflammatory cytokine, in melioidosis cases compared to healthy controls (Table 1, Fig 1). Several studies have reported the upregulated expression of TNFα during melioidosis in

human host and animal models of infection (7, 11, 12, 39). Elevated plasma concentrations of TNFα have been correlated with disease severity and mortality in septicaemic melioidosis patients (40).

CD40 ligand (CD40LG); which plays a major role in B-cell activation and development and pro-inflammatory cytokines and Lymphotoxin alpha (LTA) were down regulated in the melioidosis cohort compared to healthy controls (Table 1, fig 1). CD40L has been considered as an important mediator of sepsis, implicated in platelet-mediated activation and accumulation of neutrophils during inflammation (41, 42).

Tumor necrosis factor (ligand) superfamily 14 (TNFSF14); which plays a major role in T-cell proliferation, Tumor necrosis factor (ligand) superfamily 4 (TNFSF4); which is responsible for Th2 cell differentiation, and Tumor necrosis factor (ligand) superfamily 8 (TNFSF8); implicated in blocking Th1 responses were up regulated in the melioidosis cohort compared to other sepsis infection cases (Table 1, Fig 1). TNFSF4 was consistently upregulated in melioidosis patients compared to other sepsis cases irrespective of factors like duration of clinical symptoms, antibiotic treatment and comorbidities such as diabetes (fig 1-3). Upregulated expression of TNFSF4 (also known as OX40L), has been observed in cases of polymicrobial sepsis and autoimmune disease and has been correlated to disease severity and mortality (43). Studies have also shown that upregulated expression of TNFSF4 promoted T cell proliferation, increased expression in CD4+ T cells and production of Th2 cytokines such as IL4 (44, 45). It has also been postulated as a specific biomarker in therapeutic interventions for treatment of sepsis/ septic shock and other autoimmune diseases (43). Tumor necrosis factor (ligand) superfamily14 (TNFSF14), otherwise known as LIGHT plays a major role in systemic immune response, particularly in long term

survival of memory Th1 and Th2 cells (46). Tumor necrosis factor (ligand) superfamily 8 (TNFSF8), or CD30L, is reportedly expressed in Th2 cells and suppresses Th1 responses (46). These findings once again suggest an inclination for dominant Th2 responses during the disease progression of melioidosis.

Gene expression profile of TGFβ superfamily

Bone morphogenetic protein 6 (BMP6), inhibin beta A (INHBA) and transforming growth factor beta 1(TGFB1) showed significant upregulation in melioidosis patients compared to healthy individuals (Table 1, Fig 1). BMP3 was down regulated in other sepsis infection cases compared to healthy controls (Fig 1). BMP3, BMP4, BMP6, growth differentiation factor 2 (GDF2), INHBA, TGFB1 expressed up regulated expression in melioidosis patients compared to other sepsis cases, with BMP4 and GDF2 showing high level of gene expression (Table 2 Fig 1).

High level expression of BMP3, BMP6, TGFB1 and TGFB2 was observed in septicaemic melioidosis cohort compared to other bacterial sepsis cases (Fig 1). TGFB2 was expressed at high level in the early acute phase (<15 days of fever/clinical symptoms) melioidosis cohort compared to sepsis control (Fig 3).

TGFβ was upregulated during melioidosis infection, with increased levels being correlated to severe melioidosis in human hosts (47). Our study revealed a consistent upregulation of TGFB1 in melioidosis patients compared to other sepsis cases irrespective of factors like duration of clinical symptoms, antibiotic treatment and comorbidities such as diabetes (fig 1-3). An experimental murine model of melioidosis, revealed that an inhibition of TGF-β with a selective TGF-β antibody had a protective effect, with reduction in inflammation, reduced bacterial load and organ damage, thus indicating the role of TGF-β in pathogenesis of melioidosis (47).

Several other studies have also shown the crucial role of TGF-β in immune regulation, where it induces Foxp3, a master regulator of Tregs in naive T cells, with suppression of pro-inflammatory cytokines such as IFNγ and enhanced production of anti-inflammatory cytokines (48, 49). It has also been identified as an inducer of T17 cell differentiation (48, 50). These studies further support our findings of increased Th17 cytokine production and suppression of Th1 cytokines in melioidosis patients.

BMP3 and BMP6 were consistently up regulated in melioidosis patients compared to other sepsis cases irrespective of factors like duration of clinical symptoms, antibiotic treatment and comorbidities such as diabetes (Fig 1-3). BMP's play a major role in formation and repair of bone and cartilage, cell proliferation, differentiation and apoptosis (51). INHBA over expression has been associated with increased cell proliferation and poor disease outcome in several types of carcinomas (52, 53). Further studies are needed to elucidate the mechanisms of BMP signaling pathways and INHBA expression in relation to pathogenesis of melioidosis.

Gene expression profile of Growth factors

Platelet-derived growth factor alpha polypeptide (PDGFA)was upregulated significantly in melioidosis patients compared to healthy individuals (Table 1, Fig 1). PDGFA, thrombopoietin (THPO), ciliary neurotrophic factor (CNTF), macrophage colony stimulating factor (M-CSF or CSF1), C-fos induced growth factor or vascular endothelial growth factor D (FIGF) showed upregulation in melioidosis cases compared to other sepsis infection cases (Table 2, Fig 1). A down regulated expression in THPO was observed in other sepsis cases compared to healthy individuals (Fig 1).

PDGF is an important growth factor that plays a crucial role in blood vessel formation (angiogenesis) and regulates cell growth and differentiation. THPO stimulates the production and differentiation of megakaryocytes, thus regulating platelet production. FIGF plays an active role in angiogenesis and vascular endothelial cell growth (54). Increased expression of PDGF is seen in severe bacterial infections, implicating the role of angiogenic factors in endothelial dysfunction leading to disease pathogenesis (54). PDGF has also been suggested as a biomarker of sepsis, related to vascular endothelial damage (55). Our findings also agree with these reports as we see an increased expression of growth factors, which play a role in endothelial function.

A down regulated expression of leukemia inhibitory factor (LIF); an IL6 class cytokine that inhibits cell differentiation and a similar cytokine OSM (oncostatin M), was observed in the early acute phase melioidosis cases (<15 days fever/clinical symptoms) compared to other sepsis cases (Fig 3). M-CSF or CSF1, FIGF and PDGFA were consistently up-regulated in melioidosis patients compared to other sepsis cases irrespective of factors like duration of clinical symptoms, antibiotic treatment and comorbidities such as diabetes (Fig 1-3). Studies with experimental mice models of melioidosis have revealed a upregulation of mRNA for macrophage colony stimulating factor (CSF1 or M-CSF), granulocyte macrophage colony stimulating factor (CSF2 or GM-CSF), granulocyte colony stimulating factor (CSF3 or G-CSF) at day 3 post infection, correlating with peak bacterial load and extensive infiltration of leucocytes (56). Colony stimulating factors are glycoproteins, necessary for the survival, proliferation and differentiation of hematopoietic progenitor cells of the myeloid and erythroid lineage. M-CSF enhances the survival and activation of cells of the monocyte lineage, while GM-CSF and G-CSF increases accumulation and activation of both neutrophils and macrophages (56).While colony-stimulating

409 factors play a crucial role in innate immune responses and host defense, their high
410 level of expression during melioidosis may instead contribute to disease pathogenesis.

411 **Limitations of the study**

412 The main limitation of our study was that the melioidosis patient samples were
413 collected after start of antibiotic treatment which may affect immunocompetant cells,
414 which in turn affects the cytokine profiles studied here. Studies have shown that
415 antibiotics like meropeneum exert an immunomodulatory effect, affecting the
416 production of some cytokines in PBMC's (57). This may have been the main reason,
417 as to why we could not see any significant differential expression of some key
418 inflammatory response cytokines such as IFNγ. Duration of clinical symptoms ranged
419 from >10 days to >90 days and duration of antibiotics treatment ranged from 3 days
420 to >30 days at the time of blood collection for all the melioidosis samples. Since our
421 sample collection was nationwide, duration between patient identification/disease
422 confirmation and sampling was substantial due to logistical issues. Thus, due to
423 varying and the wide range of acute phase in each of the samples analyzed and less
424 number of samples with ≤15 days of fever/clinical symptoms duration (n=5) we could
425 not see any statistically significant differential expression of some of the
426 inflammatory response genes involved in early innate immune responses. However,
427 our results showed a consistent up regulated expression of interleukins; IL4, IL17A,
428 IL23A, IL24, interferons; IFNA1, IFNB1, TNF superfamily; TNFSF4 (OX40L), TGF
429 superfamily; BMP3, BMP6, TGFB1; other growth factors; CSF1, FIGF and PDGFA
430 in melioidosis patients compared to other sepsis cases, irrespective of comorbidities,
431 duration of fever/clinical symptoms and antibiotic treatment, indicating their

differential expression during melioidosis infection.Our findings suggest a domination of Th2 and Th17 type responses during disease pathogenesis of melioidosis.

As diabetes was seen as a major comorbidity in our experimental cohort, we analyzed our data to see if there was any significant differential expression between diabetic melioidosis cases and non-diabetic melioidosis cases. The gene expression pattern between these two groups were comparable and we could not find any statistically significant differential expression, indicating that the differential expression was largely due to melioidosis infection (Tables S1 and S2).

Conclusion

Our study revealed differential gene expression of key cytokines involved in human host responses that can distinguish melioidosis cases from sepsis infections caused by other pathogens and healthy individuals. Low level of expression of key inflammatory mediators; IL1A, IL1B and IL8 were seen in melioidosis patients in early acute phase and with septicaemia compared to other sepsis infection cases. These findings indicate that differentially expressed genes should be validated during different stages of infection for their potential as disease biomarkers for diagnostic purposes and monitoring disease progression. Our results also show an elevated expression of Th17 cytokines such as IL17, IL22 and TGF β which act as an inducer of Th17 cytokines. Th2 cytokines such as IL3, IL4 and IL13 were also upregulated along with type I interferons and TNFSF cytokines, which are known to be inducer’s of Th2 cytokines and suppressors of Th1 responses. These results may indicate a dominant Th2 and Th17 type cytokine responses, suggesting that their dysregulation may play an important role in disease pathogenesis and progression. IL17, IL23 and IL27, already implicated in therapeutic intervention of several inflammatory diseases should be

456 further investigated for their role in disease progression and therapeutic approaches in
457 melioidosis.

458 Our future studies shall be aimed at studying gene expression profiles in early and late
459 acute phases of melioidosis to evaluate candidate genes which can serve as disease
460 and diagnostic biomarkers in different stages of infection. Based on these biomarkers
461 if the antibiotic treatment regime can be adjusted it would bring benefits to the
462 patients by reducing the hospital stay. We would expand our studies further, with a
463 larger sample size in each category of sample type, focusing on specific immune
464 response genes showing differential expression, to further understand their role in
465 disease pathogenesis, susceptibility and severity associated with major comorbidities
466 such as diabetes.

467

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TABLES

Table 1. Cytokines showing significant differential expression in PBMC's of melioidosis patients (n=26) compared to healthy negative controls (n=5)

Gene	Gene description	Relative expression ratio [95% CI]	P value
IL1B	Interleukin 1 beta	2.504 [1.229 -5.100]	0.0135
IL1RN	Interleukin 1 receptor antagonist	1.62 [1.023 - 2.564]	0.0403
IL8	Interleukin 8	2.953 [1.394 -6.257]	0.0062
IL10	Interleukin 10	2.257 [1.180 - 4.319]	0.0158
IL27	Interleukin 27	4.022 [1.632 - 9.915]	0.0039
INFA5	Interferon alpha 5	0.189 [0.037 - 0.961]	0.0454
TNF	Tumour necrosis factor	2.248 [1.082 - 4.670]	0.0315
CD40LG	CD40 ligand	0.502 [0.336 - 0.752]	0.002
LTA	Lymphotoxin alpha	0.327 [0.190 - 0.565]	0.0008
BMP6	Bone morphogenetic protein 6	2.946 [1.470 - 5.904]	0.007
INHBA	Inhibin beta A	6.07 [2.652 - 13.891]	0.0002
TGFB1	Transforming growth factor 1	1.634 [1.121 - 2.383]	0.0126
PDGFA	Platelet derived growth factor alpha polypeptide	2.86 [1.444 - 5.667]	0.0066

PBMC, peripheral blood mononuclear cells; CI, confidence interval; Relative expression ratio >1.5 indicates upregulation and ≤0.5 indicates downregulation in the experimental group compared to control group; Gene targets showing significant differential expression where P-value <0.05

705 **Table 2. Cytokines showing significant differential expression in PBMC's of**

706 **melioidosis patients (n=26) compared to other sepsis cases (n=10)**

Gene symbol	Gene description	Relative expression ratio [95% CI]	P value
IL3	Interleukin 3	9.38 [1.773 - 49.626]	0.0107
IL4	Interleukin 4	6.024 [1.153- 31.479]	0.0344
IL16	Interleukin 16	1.896 [1.152 - 3.121]	0.0157
IL17A	Interleukin 17 alpha	16.32 [3.193 - 83.421]	0.0017
IL17B	Interleukin 17 beta	2.939 [1.486 - 5.811]	0.003
IL1RN	Interleukin 1 receptor antagonist	1.747 [1.091-2.796]	0.0216
IL22	Interleukin 22	3.022 [1.207 - 7.565]	0.0206
IL23A	Interleukin 23 alpha	2.792 [1.329 -5.866]	0.0092
IL24	Interleukin 24	2.991 [1.240 -7.214]	0.0173
IL27	Interleukin 27	3.089 [1.203 - 7.932]	0.0206
INFA1	Interferon alpha 1	4.034 [1.358 -11.984]	0.014
INFA5	Interferon alpha 5	0.2 [0.057 - 0.704]	0.0152
INFB1	Interferon beta 1	3.206 [1.056 - 9.735]	0.0407
TNFSF4	Tumour necrosis factor super family 4	2.349 [1.167 -4.728]	0.0202
TNFSF8	Tumour necrosis factor super family 8	1.606 [1.004 -2.571]	0.0484
TNFSF14	Tumour necrosis factor super family 14	2.353 [1.171 - 4.728]	0.0186
BMP3	Bone morphogenetic protein 3	5.305 [2.319 - 12.135]	0.0003
BMP4	Bone morphogenetic protein 4	18.765 [1.479 -238.054]	0.0271
BMP6	Bone morphogenetic protein 6	2.776 [1.214 - 6.344]	0.0192
GDF2	Growth differentiation factor 2	11.112 [1.105 -111.704]	0.0421
INHBA	Inhibin beta A	4.635 [1.205 -17.822]	0.0282

TGFB1	Transforming growth factor 1	2.006 [1.374 - 2.931]	0.0007
PDGFA	Platelet derived growth factor alpha polypeptide	2.317 [1.065 - 5.038]	0.0357
THPO	Thrombopoietin	4.213 [1.042 - 17.040]	0.0441
CNTF	Ciliary neurotrophic factor	2.222 [1.023 - 4.829]	0.0441
CSF1	Colony stimulating factor 1	2.456 [1.451 - 4.156]	0.0017
FIGF	C-fos induced growth factor	3.912 [1.561 - 9.802]	0.0049

PBMC, peripheral blood mononuclear cells; CI, confidence interval; Relative expression ratio >1.5 indicates upregulation and ≤0.5 indicates downregulation in the experimental group compared to control group; Gene targets showing significant differential expression where P-value <0.05

FIGURES

Fig 1: **Relative differential gene expression of cytokines in melioidosis patients compared to patients with sepsis infection due to other pathogens and healthy negative controls.** Significant relative gene expression changes in PBMC's from melioidosis patients (n=26) and septicaemic melioidosis patients (n=16) compared to sepsis controls (n=10) and healthy controls (n=5). Expression levels were normalized against beta actin as the reference house keeping gene. Relative expression ratio >1.5 considered as upregulation and ≤0.5 was considered as downregulation. * indicates relative expression ratio is significantly different (P<0.05)

Fig 2: **Relative differential gene expression of cytokines in diabetic melioidosis patients compared to patient with sepsis infection due to other pathogens and healthy negative controls.** Relative gene expression in PBMC's from diabetic melioidosis patients (n=17), compared to sepsis controls (n=10) and healthy controls (n=5). Expression levels were normalized against beta actin as the reference house keeping gene. Relative expression ratio >1.5 was considered as upregulation and ≤0.5 considered as downregulation. * indicates relative expression ratio is significantly different (P<0.05)

Fig 3: **Relative differential gene expression of cytokines in melioidosis patients with respect to duration of fever/clinical symptoms and antibiotics treatment, compared to patients with sepsis infection due to other pathogens.** Relative gene expression in PBMC's from melioidosis patients with ≤15 days of fever **(**n=5**)**, melioidosis patients with >15 days of fever **(**n=21**)**, melioidosis patients with ≤15 days of treatment with antibiotics **(**n=14**)**, melioidosis patients with >15 days of treatment with antibiotics **(**n=8**),** compared to sepsis controls (n=10). Expression

743 levels were normalized against beta actin as the reference house keeping gene.
744 Relative expression ratio >1.5 was considered as upregulation and ≤0.5 considered as
745 downregulation. * indicates relative expression ratio is significantly different ($P<0.05$)

746

747 **Table 1: Primer details* for Gene expression analysis**

Primer Name	Forward Sequence	Reverse Sequence	Product Size
GAPDH2	TGACAACTTTGGTATYCGTGGAAGG	AGGCAGGGATGATGTTCTGGAGAG	134 bp
18srRNA	GCTTAATTTGACTCAACACGGGA	AGCTATCAATCTGTCAATCCTGTC	69 bp
PLCE1	GCCCAAAGCAAGTGGAAAGG	TCTTCACCTGGGTTAAACATGC	700 bp
Il8	CAGAGACAGCAGAGCACACA	GGCAAAACTGCACCTTCACA	158 bp
MICB	CACCCAGGCTGCAGTTCACT	CGGGAGTCTGAGGTACGAGAA	88 bp
PSMB8	GATCTCCAGAGCTCGCTTTA	GTTCACCCGTAAGGCACTAA	200 bp
CCL5	CCATGAAGGTCTCCGCGGCAC	CCTAGCTCATCTCCAAAGAG	361 bp
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	88 bp
IL18	GCTTGAATCTAAATTATCAGTC	CAAATTGCATCTTATTATCATG	335 bp
DNMT1A	ACCGCTTCTACTTCCTCGAGGCCTA	GTTGCAGTCCTCTGTGAACACTGTGG	335 bp
DNMT3B	AATGTGAATCCAGCCAGGAAAGGC	ACTGGATTACACTCCAGGAACCGT	191 bp
HDAC1	CCAAGTACCACAGCGATGAC	TGGACAGTCCTCACCAACG	110 bp
HDAC2	TGAAGGAGAAGGAGGTCGAA	GGATTTATCTTCTTCCTTAACGTCTG	124 bp
DNMT3A	CGTTGGCATCCACTGTGAATGA	TTACACACACGCAAAATACTCCTT	551bp
IFNγ	CCAACGCAAAGCAATACATGA	CCTTTTTCGCTTCCCTGTTTTA	79 bp
TNFα	GGA GAA GGG TGA CCG ACT CA	CTG CCC AGA CTC GGC AA	70 bp
IL1β	GCAAGGGCTTCAGGCAGGCCGCG	GGTCATTCTCCTGGAAGGTCTGTGGGC	96 bp
IL4	ACTTTGAACAGCCTCACAGAG	TTGGAGGCAGCAAAGATGTC	74 bp
IL15	GTCTTCATTTTGGGCTGTTTCAGT	CCTCACATTCTTTGCATCCAGATTCT	316 bp
TLR2	GGGTCATCATCAGCCTCTCC	AGGTCACTGTTGCTAATGTAGGTG	181 bp
TLR4	CAGAGTTGCTTTCAATGGCATC	AGACTGTAATCAAGAACCTGGAGG	282 bp

PSME2	GGGAATGAGAAAGTCCTGTCC	TCAATCTTGGGGATCAGGTG	113 bp
IL12	CCAAGAACTTGCAGCTGAAG	TGGGTCTATTCCGTTGTGTC	355bp
HDAC4	GAGAGACTCACCCTTCCCG	CCGGTCTGCACCAACCAAG	240bp
PSMA5	AAGCCCATGTTGCTTTTGGG	GGCGAACGGACATAGGCTAA	112bp
PSMB2	AGAGGGCAGTGGAACTCCTT	AGGTTGGCAGATTCAGGATG	72bp
HMGB1	ACATCCAAAATCTTGATCAGTTA	AGGACAGACTTTCAAAATGTTT	122bp
HLADMB	ATGTGAAATCCTTTGGAGTCCCA	GGCATCTTTACAGAGCAGAGCAT	145bp

748 *GAPDH and 18srRNA primers are the house keeping genes. PLCE1 primer pair tested to
749 amplify 700bp genomic region of PLCEI is used as genomic DNA control.

750

751 Table 2. mRNA expression in PBMC's of melioidosis patients (n=30) compared to other
752 sepsis cases (n=10) and healthy negative controls (n=10)

753

	Melioidosis vs Healthy controls		Melioidosis vs Sepsis controls		Sepsis cases vs Healthy controls	
Gene	Relative expression ratio [95% CI]	P value	Relative expression ratio [95% CI]	P value	Relative expression ratio [95% CI]	P value
HMGB1	0.83[0.444,1.539]	0.5324	0.26[0.132,0.510]*	0.0005	3.18[1.539,6.578]*	0.0036
IL6	1.56[0.670,3.623]	0.2912	0.89[0.360,2.189]	0.7867	1.76[0.710,4.344]	0.2079
IL8	1.65[0.727,3.733]	0.2187	0.43[0.169,1.080]	0.0699	3.86[1.411,10.540]*	0.0114
IL1β	1.33[0.554,3.205]	0.5005	0.77[0.258,2.320]	0.6254	1.72[0.516,5.738]	0.3545
IFNγ	1.34[0.826,2.189]	0.2174	0.88[0.496,1.572]	0.6522	1.52[0.800,2.899]	0.1864
TNFα	1.22[0.705,2.124]	0.4573	0.81[0.408,1.598]	0.5173	1.52[0.741,3.100]	0.2365
IL15	1.31[0.751,2.296]	0.3254	0.57[0.258,1.279]	0.1610	2.29[1.025,5.103]*	0.0443
IL4	1.78[0.115,27.531]	0.3533	4.09[1.178,14.173]*	0.0366	0.44[0.065,2.942]	0.2329
TLR2	1.16[0.666,2.034]	0.5844	0.44[0.196,1.009]	0.0522	2.62[1.182,5.803]*	0.0212
TLR4	0.97[0.532,1.777]	0.9247	0.37[0.141,0.974]*	0.0448	2.62[1.025,6.700]*	0.0450
MICB	0.95[0.533,1.699]	0.8607	0.33[0.183,0.582]*	0.0006	2.92[1.534,5.545]*	0.0026
HLADMB	0.84[0.502,1.396]	0.4814	0.69[0.396,1.204]	0.1809	1.21[0.682,2.156]	0.4897
PSMB2	0.88[0.513,1.519]	0.6403	0.25[0.121,0.508]*	0.0008	3.56[1.708,7.420]*	0.0021
PSME2	1.24[0.763,1.998]	0.3757	0.38[0.195,0.726]*	0.0061	3.28[1.684,6.389]*	0.0017
PSMB8	0.83[0.456,1.519]	0.5356	0.27[0.131,0.565]*	0.0014	3.06[1.443,6.480]*	0.0060
PSMA5	0.75[0.421,1.328]	0.3086	0.84[0.412,1.706]	0.6102	0.89[0.448,1.778]	0.7301
DNMT1A	0.66[0.376,1.171]	0.1494	0.51[0.211,1.213]	0.1171	1.31[0.542,3.169]	0.5224
DNMT3A	0.68[0.416,1.097]	0.1087	0.68[0.356,1.300]	0.2284	0.99[0.533,1.849]	0.9793
DNMT3B	1.07[0.636,1.814]	0.7770	0.22[0.088,0.539]*	0.0040	4.94[1.948,12.503]*	0.0031
HDAC1	0.78[0.556,1.095]	0.1441	0.50[0.353,0.719]*	0.0006	1.55[1.059,2.267]*	0.0266
HDAC2	0.68[0.377,1.212]	0.1799	0.28[0.126,0.642]*	0.0048	2.38[1.047,5.403]*	0.0398
HDAC4	0.99[0.588,1.652]	0.9549	0.64[0.322,1.270]	0.1880	1.54[0.786,3.025]	0.1911

754

755 PBMC, peripheral blood mononuclear cells; CI, confidence interval; Relative expression ratio
756 >1 indicates up regulation and ≤0.5 indicates down regulation in the experimental group
757 compared to control group; * indicates statistically significant differential expression where
758 P-value <0.05, P values calculated by paired t-tests

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760

761

Figure 1. Relative expression of genes involved in immune response and epigenetic regulation in melioidosis patients compared to patients with sepsis infections caused by other pathogens and healthy controls. Statistically significant differential expression of genesin PBMC's from melioidosis patients (n=30), septicaemic melioidosis patients (n=18) compared to sepsis controls (n=10). Significant differential expression was not observed among melioidosis patients compared to healthy controls (n=10) while significant differential expression was observed among sepsis controls and the healthy controls. Expression levels were normalized against 18srRNA. Relative expression ratio >1.5 considered as up regulation and ≤0.5 considered as down regulation, with P<0.05 considered statistically significant.

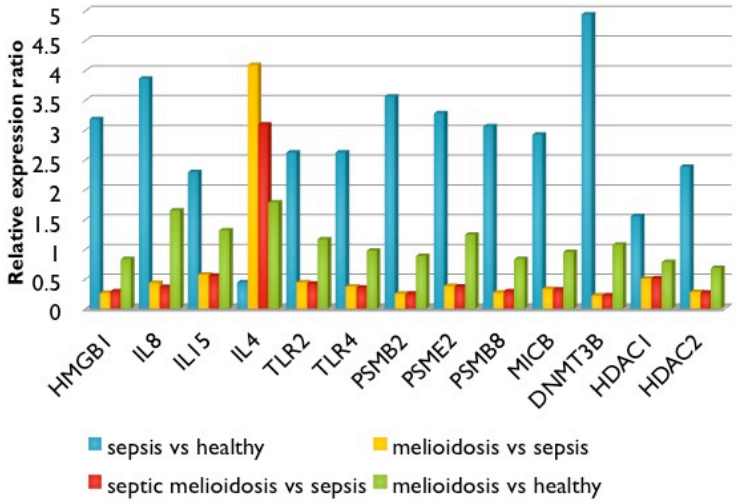


Figure 2. Relative expression of genes involved in immune responses and epigenetic regulation in melioidosis patients compared to patients with sepsis infections caused by other pathogens, in relation to duration of fever/clinical symptoms and antibiotics treatment. Differential gene expression in PBMC's from melioidosis patients with ≤15 days of fever **(**n**=**4**)**, melioidosis patients with >15 days of fever **(**n**=**25**)**, melioidosis patients with ≤15 days of treatment with antibiotics **(**n**=**15**)**, melioidosis patients with >15 days of treatment with antibiotics **(**n**=**12**)** compared to sepsis controls **(**n**=**10**),** did not change due to the duration of fever or duration of treatment with antibiotics. Expression levels were normalized against 18srRNA. Relative expression ratio >1.5 considered up regulated and ≤0.5 considered as down regulation, with P<0.05 considered statistically significant.

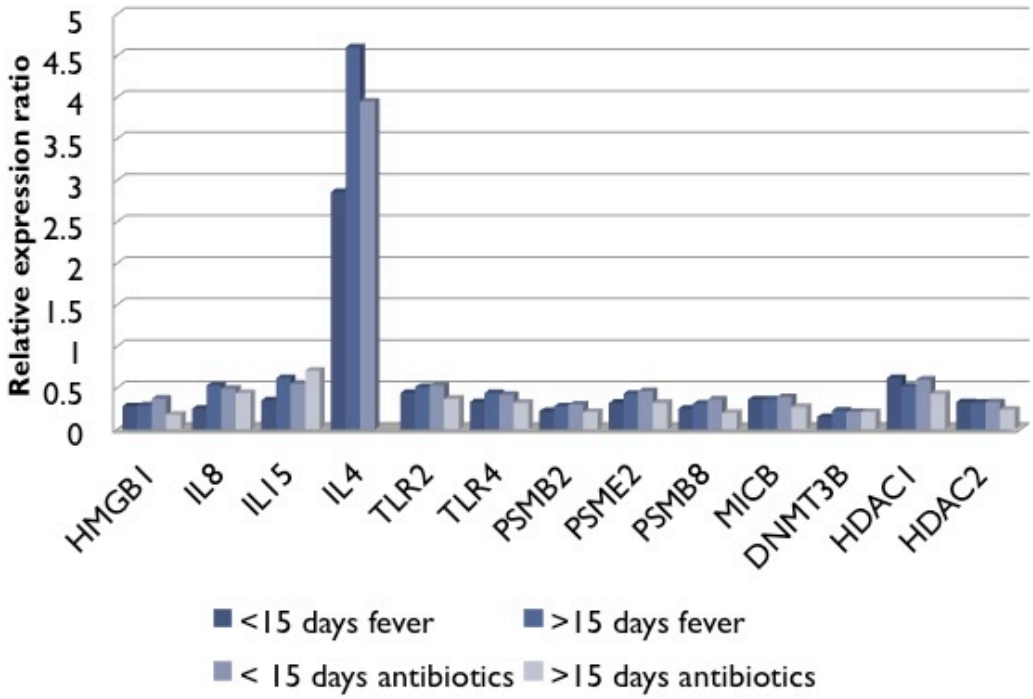
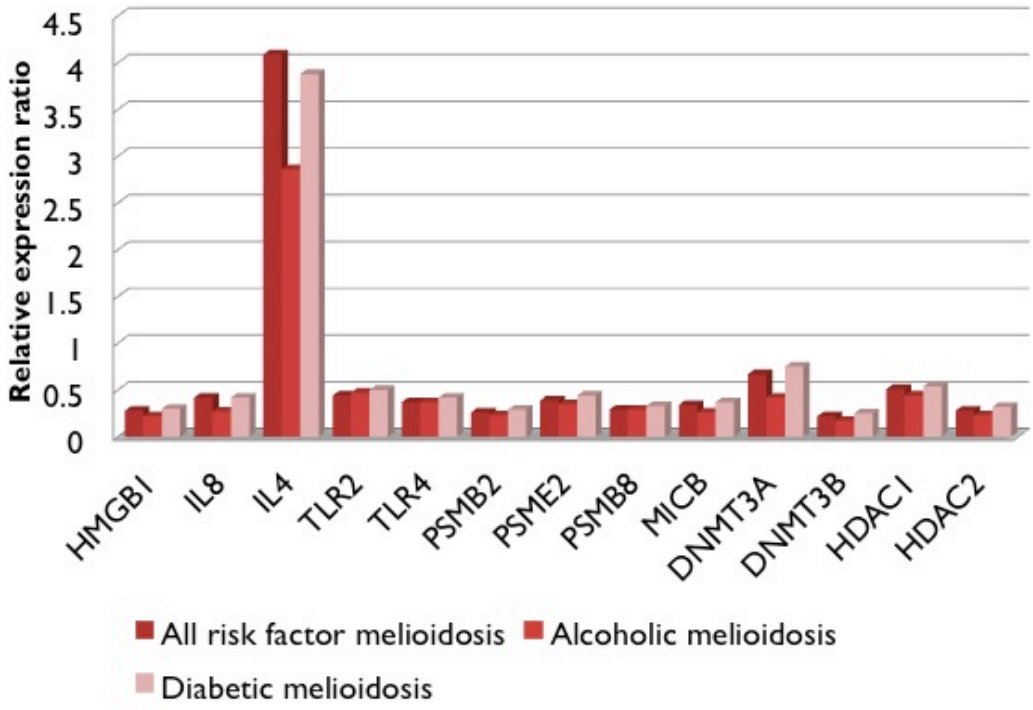


Figure 3. Relative expression of genes involved in immune responses and epigenetic regulation in melioidosis patients compared to patients with sepsis infections caused by other pathogens, in relation to associated comorbidities. Differential gene expression in PBMC's from melioidosis patients with risk factors (n=27), alcoholic melioidosis patients (n=8), diabetic melioidosis patients (n=20), compared to sepsis controls (n=10), did not change among patientss presented with different risk factors for melioidosis. Expression levels were normalized against 18srRNA. Relative expression ratio >1.5 was considered as up regulated and ≤0.5 was considered as down regulated, with $P<0.05$ considered statistically significant.



819 **S1: List of gene targets investigated**

Abbreviated name	Full Name	Biological Role
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Involved in several metabolic processes such as glycolysis
18SrRNA	18S ribosomal RNA	Structural RNA and basic component of eukaryotic cells
PLCE1	1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase epsilon-1	Involved in intracellular responses involving cell growth and differentiation
IL1β	Interleukin 1 beta	Pro-inflammatory cytokine, important mediator of the inflammatory response, involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis.
IL4	Interleukin 4	Anti-inflammatory cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells, decreasing production of Th1 cells. It is a key regulator in humoral and adaptive immunity playing a major role in stimulation of activated B-cell and T-cell proliferation.
IL6	Interleukin 6	Pro-inflammatory cytokine and an anti-inflammatory myokine. It is secreted by T cells and macrophages to stimulate immune response
IL8	Interleukin 8	Chemokineassociated with inflammation, induces chemotaxis in target cells, mainly involved in neutrophil recruitment and degranulation.
IL10	Interleukin 10	Anti-inflammatory cytokine involved in immune regulation and inflammation. It down regulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL-10 can block NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway.
IL12	Interleukin 12	Pro-inflammatory cytokine involved in the differentiation of naive T cells into Th1 cells. It is involved in stimulation and growth T cells and in production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from T cells and natural killer (NK) cells. It reduces IL-4 mediated suppression of IFN-γ.
IL15	Interleukin 15	Pro-inflammatory cytokine which regulates T and natural killer (NK) cell activation and proliferation.

IL18	Interleukin 18	Pro-inflammatory cytokine involved in inflammation and cell-mediated immunity along with IL12
CCL5	Chemokine (C-C motif) ligand 5. Also known as RANTES (regulated on activation, normal T cell expressed and secreted).	Chemokine, which is chemotactic for T cells, eosnophils and basophils, plays an active role in recruiting leukocytes into inflammatory sites
IFNγ	Interferon gamma	Pro-inflammatory cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections. It is an important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression.
TNFα	Tumor necrosis factor alpha	Pro-inflammatory cytokine involved in systemic inflammation and immune regulation
HMGB1	High mobility group box 1 protein, also known as high-mobility group protein 1 (HMG-1)	Cytokine mediator of inflammation secreted by activated macrophages and monocytes
TLR2	Toll-like receptor 2	Plays a fundamental role in pathogen recognition and activation of innate immunity. This gene is expressed abundantly in peripheral blood leukocytes and mediates host response to gram-positive bacteria.
TLR4	Toll-like receptor 4	Plays a fundamental role in pathogen recognition and activating the innate immune system. It is well-known for recognizing lipopolysaccharide (LPS), a component present in many gram-negative bacteria, thus mediates its host responses
MICB	MHC class I polypeptide-related sequence B	Heavily glycosylated protein which is a ligand for the NKG2D type II receptor. Binding of the ligand activates the cytolytic response of natural killer (NK) cells, CD8 alpha beta T cells, and gamma delta T cells which express the receptor.
PSMB8	Proteasome subunit beta type-8 also known as 20S proteasome subunit beta-5i	Forms a pivotal component for the Ubiquitin-Proteasome System (UPS) involved in protein ubiquitination and subsequent proteolysis and degradation which are important mechanisms in the regulation of the cell cycle, cell growth and differentiation, gene transcription, signal transduction and apoptosis. During the antigen processing for the major histocompatibility complex (MHC) class-I, the proteasome is the major degradation machinery that degrades the antigen and present the resulting peptides to cytotoxic Tcells.

PSMB2	Proteasome subunit beta type-2 also known as 20S proteasome subunit beta-4	Forms a pivotal component for the UPS involved in protein ubiquitination and subsequent proteolysis and degradation which are important mechanisms in the regulation of the cell cycle, cell growth and differentiation, gene transcription, signal transduction and apoptosis. It is also involved in processing of class I MHC peptides
PSME2	Proteasome activator complex subunit 2	Process class I MHC peptides
PSMA5	Proteasome subunit alpha type-5 also known as 20S proteasome subunit alpha-5	Process class I MHC peptides
HLADMB	HLA class II histocompatibility antigen, DM beta chain	Plays a central role in the peptide loading of MHC class II molecules by helping to release the CLIP (class II-associated invariant chain peptide) molecule from the peptide binding site, thus playing a major role in MHC class II antigen presentation pathway
DNMT1A	DNA methyltransferase 1A	Enzyme catalyzes the transfer of methyl groups to specific CpG structures in DNA (DNA methylation). Considered to be the key maintenance methyl transferase in mammals. predominantly methylates hemi methylated CpG di-nucleotides in the mammalian genome.
DNMT3A	DNA (cytosine-5)-methyl transferase 3A	DNA methyl transferaseresponsible for de novo DNA methylation.
DNMT3B	DNA (cytosine-5-)-methyl transferase 3 beta	DNA methyl transferase responsible for de novo DNA methylation.
HDAC1	Histone deacetylase 1	Class I histone deacetylase, playing a key role in the regulation of eukaryotic gene expression
HDAC2	Histone deacetylase 2	Class I histone deacetylase, playing a key role in transcriptional regulation and regulation of eukaryotic gene expression
HDAC4	Histone deacetylase 4	Class II histone deacetylase, playing a key role in transcriptional regulation and regulation of eukaryotic gene expression

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822 **S2: Primer details for Gene expression analysis**

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Primer Name	Forward Sequence	Reverse Sequence	Product Size
GAPDH[a]	TGACAACTTTGGTATYCGTGGAAGG	AGGCAGGGATGATGTTCTGGAGAG	134 bp
18srRNA[a]	GCTTAATTTGACTCAACACGGGA	AGCTATCAATCTGTCAATCCTGTC	69 bp
PLCE1[b]	GCCCAAAGCAAGTGGAAAGG	TCTTCACCTGGGTTAAACATGC	700 bp
IL8	CAGAGACAGCAGAGCACACA	GGCAAAACTGCACCTTCACA	158 bp
MICB	CACCCAGGCTGCAGTTCACT	CGGGAGTCTGAGGTACGAGAA	88 bp
PSMB8	GATCTCCAGAGCTCGCTTTA	GTTCACCCGTAAGGCACTAA	200 bp
CCL5	CCATGAAGGTCTCCGCGGCAC	CCTAGCTCATCTCCAAAGAG	361 bp
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	88 bp
IL18	GCTTGAATCTAAATTATCAGTC	CAAATTGCATCTTATTATCATG	335 bp
DNMT1A	ACCGCTTCTACTTCCTCGAGGCCTA	GTTGCAGTCCTCTGTGAACACTGTGG	335 bp
DNMT3B	AATGTGAATCCAGCCAGGAAAGGC	ACTGGATTACACTCCAGGAACCGT	191 bp
HDAC1	CCAAGTACCACAGCGATGAC	TGGACAGTCCTCACCAACG	110 bp
HDAC2	TGAAGGAGAAGGAGGTCGAA	GGATTTATCTTCTTCCTTAACGTCTG	124 bp
DNMT3A	CGTTGGCATCCACTGTGAATGA	TTACACACACGCAAAATACTCCTT	551 bp
IFNγ	CCAACGCAAAGCAATACATGA	CCTTTTTCGCTTCCCTGTTTTA	79 bp
TNFα	GGA GAA GGG TGA CCG ACT CA	CTG CCC AGA CTC GGC AA	70 bp
IL1β	GCAAGGGCTTCAGGCAGGCCGCG	GGTCATTCTCCTGGAAGGTCTGTGGGC	96 bp
IL4	ACTTTGAACAGCCTCACAGAG	TTGGAGGCAGCAAAGATGTC	74 bp
IL15	GTCTTCATTTTGGGCTGTTTCAGT	CCTCACATTCTTTGCATCCAGATTCT	316 bp
TLR2	GGGTCATCATCAGCCTCTCC	AGGTCACTGTTGCTAATGTAGGTG	181 bp
TLR4	CAGAGTTGCTTTCAATGGCATC	AGACTGTAATCAAGAACCTGGAGG	282 bp

PSME2	GGGAATGAGAAAGTCCTGTCC	TCAATCTTGGGGATCAGGTG	113 bp
IL12	CCAAGAACTTGCAGCTGAAG	TGGGTCTATTCCGTTGTGTC	355 bp
HDAC4	GAGAGACTCACCCTTCCCG	CCGGTCTGCACCAACCAAG	240 bp
PSMA5	AAGCCCATGTTGCTTTTGGG	GGCGAACGGACATAGGCTAA	112 bp
PSMB2	AGAGGGCAGTGGAACTCCTT	AGGTTGGCAGATTCAGGATG	72 bp
HMGB1	ACATCCAAAATCTTGATCAGTTA	AGGACAGACTTTCAAAATGTTT	122 bp
HLADMB	ATGTGAAATCCTTTGGAGTCCCA	GGCATCTTTACAGAGCAGAGCAT	145 bp

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825 [a]GAPDH and 18srRNA primers are house keeping genes. [b]PLCE1 primer pair tested to
826 amplify 700bp genomic region of PLCEI is used as genomic DNA control.

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