MELIOIDOSIS IN SOUTH-EAST ASIA: CONTRIBUTION TO A FEVER COHORT, AND IN VITRO STUDIES OF MAIT CELL FUNCTION

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Abstract

Melioidosis is a neglected tropical disease caused by the Gram-negative bacterium *Burkholderia pseudomallei* (BP). Diabetes mellitus (DM) is a major risk factor. A mathematical model estimated 1,650,000 melioidosis cases in 2015 worldwide with 16,931 cases in Bangladesh, but the disease is rarely diagnosed in Bangladesh. This thesis set out to determine whether melioidosis is a significant cause of fever in Bangladesh, and to characterise the human mucosal-associated invariant T-cell (MAIT) response to BP and other Gram-negative bacteria.

We prospectively determined the aetiology of fever in 201 adult patients in Dhaka, Bangladesh, and found melioidosis to be the joint commonest cause (with *E. coli*) of bacteraemia. We also noticed high rates of antimicrobial resistance in Gram-negative infections.

We tracked the phenotype, activation and function of MAIT cells during acute melioidosis and other Gram-negative infections. MAIT cells responded rapidly to BP, releasing IFN-γ via both MR1 and cytokine-dependent pathways. The frequency and IFN-γ expression by MAIT cells following BP stimulation was significantly reduced in acute melioidosis patients compared to endemic controls, and significantly reduced in acute patients who died compared to survivors. We then examined the effect of DM on the MAIT cell response. In the endemic control population, IFN-γ expression was reduced in DM compared to non-DM. This was not explained by differences in activation or exhaustion markers (PD-1 and TIM-3) between groups. Finally, we found IFN-γ production by MAIT cells was significantly lower in the Gram-negative sepsis patients compared to healthy controls.

In conclusion, we have demonstrated that melioidosis is one of the commonest causes of community-acquired bacteraemia in Bangladesh. We have also revealed a potential key role for MAIT cells in survival in acute melioidosis, and their significance in host defence against other Gram-negative bacteria.

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.

Dedicated to

My Beautiful and Caring Wife

Nazia

And

My two Gorgeous and Intelligent Daughters

Nawal and Neemat

Table of Contents

Contents	Page No
Abstract	2
Thesis contributions	10
Abbreviation	11
1. Introduction	16
1.1: Scenario of Sepsis: Global and in South Asia	16
1.2. Gram-negative bacteria causing sepsis	19
1.3: Melioidosis	22
1.3.1. Epidemiology	23
1.3.1.1. Presence in the environment	23
1.3.1.2. Global burden of melioidosis cases	25
1.3.1.3. Burden of melioidosis in Bangladesh	27
1.3.1.4. Risk factors	28
1.3.1.5. Clinical manifestations of melioidosis	29
1.3.1.6. Diagnosis of melioidosis	31
1.3.2. The immunopathogenesis of melioidosis	34
1.3.3. The innate immune response to BP	36
1.3.4. Adaptive immune response	38
1.3.5. The inflammatory response	42
1.4: Mucosal-associated invariant T-cell (MAIT)	42
1.4.1. History of discovery, its receptor and antigen	42
1.4.2. MAIT cell development	45
1.4.3. MAIT cell distribution	46
1.4.4. MAIT cell phenotype and effector functions	48
1.4.5. The role of MAIT cells in health and disease	50
1.5. Immune pathogenesis of Gram-negative sepsis	52
1.6. DM and Gram-negative bacterial infection	54
2. Methods	57
2.1. Is melioidosis a significant cause of febrile illness in Dhaka, Bangladesh?	57
2.1.1. Study settings	57
2.1.2. Study set-up	58
2.1.3. Ethical permissions	58

2.1.4. Microbiology laboratory	59
2.1.5. Study population	60
2.1.6. Sample size calculation	62
2.1.7. Recruitment	62
2.1.8. Screening and Eligibility Assessment	63
2.1.9. Informed Consent	63
2.1.10.Study visit	63
2.1.11. Sample Handling	65
2.1.12. Data management	66
2.1.13. Quality assurance procedure	67
2.1.14. Statistics and analysis	67
2.1.15. Funding	67
2.2. Stimulation of MAIT cells with BP and other Gram-negative bacteria	68
2.2.1. A. Study subject (BP infection)	68
2.2.1. B. Study subjects (Gram-negative infection)	69
2.2.2. Ethical permissions	69
2.2.3. Peripheral blood mononuclear cell isolation	70
2.2.4. THP-1 cell maintenance and propagation	71
2.2.5. Co-culture and intracellular staining	71
2.2.6. Gram-negative bacteria/ antigen	72
2.2.7. Flow cytometry staining and antibodies/dyes for FACS	73
2.2.8. Statistical analysis	75
3. Is melioidosis an important cause of febrile admissions in Bangladesh?	76
3.1. Introduction	76
3.2. Aims	77
3.3. Results	78
3.3.1. Patient enrolment profile and status of different mode of cultures	78
3.3.2. Baseline socio-demographic characteristics of the patients admitted with fever in DMCH and BIRDEM hospital	79
3.3.3. Clinical features, comorbidities and clinical outcome of acute febrile patients	81

3.3.4. Baseline laboratory parameters of the patients admitted with acute fever	84
3.3.5. Clinical diagnosis and laboratory confirmation of the cases	86
3.3.6. Demographic and clinical characteristics of the Melioidosis cases	88
3.4. Discussion	90
3.5. Limitations	96
3.6. Conclusion	97
4. MAIT cells are important in host defence against BP infection	98
4.1. Introduction	98
4.2. Aims	99
4.3. Results	99
4.3.1. MAIT cell responses to BP	99
4.3.2. Optimizing the concentration of BP and *E. coli.*	102
4.3.3. Gating strategy to identify MAIT cells in different T cell populations	104
4.3.4. MAIT cells and MAIT-IFN-γ expression are reduced in acute melioidosis patients who died compared to survivors	105
4.3.5. MAIT cells and MAIT-IFN-γ production are reduced in acute melioidosis patients compared to healthy endemic controls	107
4.3.6. The activation marker CD69 is significantly higher in sepsis patients compared to different controls, without any difference in GzmB expression	109
4.3.7. IFN-γ expression upon BP stimulation is mediated by both MR1 and cytokine dependent pathways, with greater predominance of cytokine-dependency	111
4.4. Discussion	113
4.5. Limitations	119
4.6. Conclusion	120
5. Are MAIT cells important for host defence in people with or without DM against BP infection?	121
5.1. Introduction	121
5.2. Aims	122
5.3. Results	123
5.3.1. IFN-γ release by MAIT cells is reduced in people with DM compared to non-DM amongst the endemic population.	123

5.3.2. No difference was found in the frequency of MAIT cells and MAIT-IFN-γ expression in recovered melioidosis patients comparing between DM and Non DM.	125
5.3.3. No difference was revealed for the expression of activation marker CD69 and cytotoxic effector molecule Gr B expression between DM and non-DM populations	127
5.3.4. There was no difference between DM and non-DM groups amongst the endemic population in terms of exhaustion marker PD-1 and TIM-3 expression.	129
5.4. Discussion	131
5.5 Limitations	134
5.6. Conclusion	135
6. MAIT cells in acute infection with different Gram-negative bacteria	136
6.1 Introduction	136
6.2. Aims	137
6.3. Results	137
6.3.1. Identification of optimum concentration of Klebsiella pneumoniae, Pseudomonas aeruginosa and Salmonella Typhi.	137
6.3.2. IFN-γ expression by MAIT cells is reduced in patients infected with different Gram-negative bacteria compared to healthy controls.	140
6.3.3. The cytotoxic effector molecule Gr B is significantly higher in Gram-negative patients compared to healthy controls, without any difference in activation.	143
6.4. Discussion	145
6.5. Limitations	148
6.6. Conclusion	149
7. Conclusion	150
8. Limitations	154
9. Future Work	155
10. References	158
Annexes	
Annex 1. Ethical permission from OXTREC for Bangladesh project	185
Annex 2. Ethical permission from DMC for Bangladesh project	187

Annex 3. Ethical permission from BIRDEM for Bangladesh project	188
Annex 4. Informed consent sheet of Bangladesh project	189
Annex 5. Patient information sheet of Bangladesh project	193
Annex 6. Case record form of Bangladesh project	197
Annex 7. letter to the control participants diagnosed as DM	202
Annex 8. SOP for PBMC isolation	203
Annex 9. SOP for cryopreservation and thawing of cell	205
Annex 10. SOP for *E. coli* preparation (culture, fixation and counting)	208
Annex 11: SOP for B. pseudomallei preparation (culture, fixation and counting)	211
Annex 12: Protocol for Flow cytometry staining: extracellular (ECS) and intracellular (ICS) for MAIT cells functional response	212
Annex 13: Protocol for Flow cytometry staining: extracellular (ECS) and intracellular (ICS) for MAIT cells activation, phenotype and exhaustion response	217
Annex 14: SOP for *S.* Typhi preparation (culture, fixation and counting)	223
Annex 15: SOP for *K. pneumoniae* preparation (culture, fixation and counting)	226
Annex 16: SOP for *P. aeruginosa* preparation (culture, fixation and counting)	229
Annex 17: Choropleth map illustrating the frequency of culture confirmed melioidosis cases by district in Bangladesh	232
Annex 18: Table: Antimicrobial resistance pattern of all culture confirmed cases (n=21)	233

Thesis contributions

All work presented in this thesis is my own, except as follows:

Chapter 2.1

Mr Bikash Chondro Das (Research assistant, Dhaka, Bangladesh)

Helped to collect adult febrile patient's clinical and demographic information in Dhaka.

Chapter 2.1

Mr Md. Rokibul Hasan (Research assistant, Dhaka, Bangladesh)

Helped to isolate PBMC, serum and plasma from Gram-negative culture confirmed patients in Dhaka.

Chapter 2.2

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Culture, fixation and counting of *Burkholderia pseudomallei* strain

Chapter 2.2

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Culture, fixation and counting of *Salmonella* Typhi strain

Chapter 2.2

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Provided *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strain.

Chapter 5

Dr Barbara Kronsteiner-Dobramysl (postdoctoral research assistant, Dunachie Group, Medawar, Oxford, UK)

Provided figures for CD69 and PD-1 expression mentioned in 5.3 and 5.4

List of Abbreviations:

AMR:	Anti-microbial resistance
APC:	Antigen presenting cell
AT:	Adipose tissue
ATCC:	American Type Culture Collection
BIRDEM:	Bangladesh institute of research and rehabilitation in Diabetes, Endocrine, and Metabolic disorders
BMI:	Body mass index
BP:	*Burkholderia pseudomallei*
BSMMU:	Bangabandhu Sheikh Mujib Medical University
CDC:	Centre for Disease Control
CKD:	Chronic Kidney Disease
CLSI:	Clinical and Laboratory Standards Institute
COPD:	Chronic obstructive pulmonary disease
CPE:	Carbapenamase producing Enterobacteriaceae
CRF:	Case record form
DALY:	Disability-adjusted life years
DC:	Dendritic cells
DM:	Diabetes mellitus
DMC:	Dhaka medical college hospital
DMSO:	Dimethyl sulfoxide
DN:	Double negative

DP: Double positive

E. coli: *Escherichia coli*

Elispot: Enzyme-Linked ImmunoSpot

EMO: Emergency Medical Officer

ESBL: Extended spectrum beta-lactamases

FACS: Fluorescence-activated cell sorting

FCS: Fetal calf serum

GCP: Good clinical practice

GCS: Glasgow Coma Scale

GzmB: Granzyme B

HCV: Hepatitis C virus

HIC: High-income countries

HTLV-1: Human T lymphotropic virus type 1

ICT: Immunochromogenic cassette test

ICU: Intensive care unit

IFA: Immunofluorescence Assay

IFN-γ: Interferon- gamma

IHA: Indirect haemagglutination assay

iNKT: invariant NK T

IR: Insulin resistance

K. pneumoniae: *Klebsiella pneumoniae*

LBP: LPS-binding protein

LFI: Lateral Flow Immunoassay

LMIC: Low and Middle income countries

LPS: Lipopolysaccharide

MAIT: Mucosal-associated invariant T-cell

MDR: Multi-drug resistant

MHC: Major Histocompatibility Complex

MLST: Multi-Locus Sequence Type

MOI: Multiplicity of infection

MORU: Mahidol Oxford Research Unit

MR1: MHC class I-related protein

MRSA: Methicillin resistant *Staphylococcus aureus*

MS: Multiple sclerosis

NARST: Nalidixic Acid Resistant *Salmonella* Typhi

NCD: Non-Communicable Disease

NK: Natural killer

OVG: Oxford Vaccine Group

OXTREC: Oxford Tropical Medicine Research Ethics Committee

P. aeruginosa: *Pseudomonas aeruginosa*

PAMP: Pathogen Associated Molecular Patterns

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate Buffer Saline

PCR: Polymerase Chain Reaction

PD-1: Programmed cell death protein 1

PF: Paraformaldehyde-fixed

PI: Principal investigator

PIS: Patients information sheet

PLZF: Promyelocytic leukaemia zinc finger protein

PRR: Pattern recognition receptors

PUO: Pyrexia of unknown origin

RA: Research assistant

REC: Research Ethics Committee

RP: Resident physician

RS: Resident surgeon

RTI: Respiratory Tract Infection

S. typhi: *Salmonella* Typhi

SEB: *Staphylococcal* enterotoxin B

SIRS: Systemic Inflammatory Response Syndrome

SLE: Systemic lupus erythematosus

SOFA: Sequential (or Sepsis-related) Organ Failure Assessment

T3SS: Type III Secretion System

T6SS: Type VI Secretion System

TB: *Mycobacterium tuberculosis*

TCR: T-cell receptor

TH1: CD4+ T helper 1

TLR: Toll-like receptors

TNFα: Tumour necrosis factor α

T-reg: Regulatory T cells

UTI: Urinary tract infection

WBC: White blood cell

WHO: World Health Organization

1. Introduction:

1.1: Scenario of Sepsis: Global and in South Asia

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection [bacterial, viral and fungal] (Shankar-Hari et al., 2016). It is a common and serious problem in both high-income countries (HIC) and low and middle-income countries (LMIC), and often requires intensive care unit (ICU) admission (Gotts and Matthay, 2016). Sepsis is difficult to define due to an overlap between infective and non-infective critical illness. The definition has recently been revised from the systemic inflammatory response syndrome (SIRS) definition (Levy et al., 2003) to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) (Shankar-Hari et al., 2016), as shown in Figure 1.

Category	Definition
PREVIOUS DEFINITIONS	
SIRS (systemic inflammatory response syndrome)	Two of the following: • Temperature >38°C or <36°C • Heart rate > 90 beats/min • Respiratory rate >20 breaths/min or arterial carbon dioxide pressure <32 mm Hg • White blood cell count >12×10⁹/L or <4×10⁹/L
Sepsis	SIRS with infection (presumed or proven)
Severe sepsis	Sepsis with evidence of acute organ dysfunction (hypotension, lactic acidosis, reduced urine output, reduced PaO_2/FIO_2 ratio, raised creatinine or bilirubin, thrombocytopenia, raised international normalized ratio)
Septic shock	Sepsis with persistent hypotension after fluid resuscitation
REVISED DEFINITIONS	
Sepsis	Life threatening organ dysfunction* caused by a dysregulated host response to infection
Septic shock	Sepsis and vasopressor therapy needed to increase mean arterial pressure to ≥65 mm Hg and lactate to >2 mmol/L despite adequate fluid resuscitation

Figure 1.1: Previous and recently revised definitions of sepsis and related syndromes. *As assessed by an acute change of ≥2 points in the sequential organ failure assessment (SOFA) score components: partial pressure of oxygen in arterial blood/fractional inspired oxygen (PaO_2/FIO_2) ratio, Glasgow coma scale (GCS), mean arterial pressure, vasopressor use, serum creatinine or urine output, bilirubin, and platelet count (Gotts and Matthay, 2016). **N.B.** For clarification, the revised definition of septic shock is “Sepsis and vasopressor therapy needed to increase mean arterial pressure to ≥ 65mmHg, and having a lactate of >2 mmol/L despite having adequate fluid resuscitation.

Over the last few decades, the incidence of sepsis has gradually increased because of resistance against common antibiotics, increasing age of the population and other factors (Gotts and Matthay, 2016). However, in-hospital mortality decreased, most likely due to the availability of third-generation antibiotics, improvement in management strategies (e.g. early diagnosis, faster “door-to-needle” time for antibiotics), and improvements in supportive care (e.g. fluid management and respiratory support). The global epidemiology and mortality data of sepsis varied between regions because of differences in a number of factors including the aetiology of sepsis, antimicrobial resistance (AMR) pattern, provision of public health services, availability of ICU facilities, and economic status (Rudd et al., 2018). Data from LMICs are very limited even though 80% of the global mortality caused by infection occurs in these countries (Cheng et al., 2008, Schultz et al., 2017).

An estimation primarily based on HIC data suggested there are around 31.5 million sepsis patients worldwide every year, with an estimated 5.3 million deaths annually (Daniels et al., 2018, Fleischmann et al., 2016). The same model estimated the population-based incidence of sepsis is as high as 176 to 380 cases per 100,000 population (Daniels et al., 2018, Martin et al., 2003). Among European countries the annual incidence of sepsis has been estimated at 90.4 cases per 1000,000 population (Daniels et al., 2018). In the United States and U.K, the prevalence ranged between 0.4/1000 and 1/1000 (Angus et al., 2001, Brun-Buisson et al., 2004, Gotts and Matthay, 2016, Harrison et al., 2006, Martin et al., 2003, van Gestel et al., 2004).

Epidemiological and mortality data on sepsis in LMIC is clearly understudied (Cheng et al., 2008). Adult sepsis is neglected compared to neonatal or paediatric sepsis, although the majority of deaths and disability-adjusted life years (DALY) loss

occurring in LMIC are due to infection-related sepsis (Cheng et al., 2008). The main reported infective syndromes resulting in death in LMIC are meningitis, respiratory tract infections (RTI) and others [malaria, dengue, rabies, tetanus, fungal etc.] (Schultz et al., 2017). Population-based data on the prevalence of sepsis in South and Southeast Asia is limited. A multinational multicentre cross-sectional study was recently conducted in Southeast Asia involving Thailand, Vietnam and Indonesia (Limmathurotsakul, 2017). This study was performed in 13 hospitals and identified 33.4% (1582/4736) patients (both adult and children) admitted with sepsis. The prevalence of adult sepsis in this study was 17.2% (815/4736) and 28-day mortality was 13% (108/804) (Limmathurotsakul, 2017). In 2009, a large prospective study (MOSAIC) on severe sepsis in adults was conducted in 16 South and Southeast Asian countries enrolling patients from 150 ICUs (Phua et al., 2011). The study identified differences in the prevalence of severe sepsis admission within the region. The prevalence was 10.8% in Bangladesh, 53.9% in Nepal, 5.5% in India, 28.3% in Pakistan, 9.1% in China, 6.3% in Indonesia and 10.7% in Vietnam (Phua et al., 2011). The mortality was divided into three groups, low, middle and high-income countries. The in-hospital mortality was 46.6% and 50% for low and middle-income countries (where all the above-mentioned countries correspond) respectively. The overall mortality was 44.5% in this cohort. One study in Bangladesh identified 95 (41% of total admission) severe sepsis patients admitted within six months (Ahmed et al., 2015). Another prospective study in 15 ICUs in Dhaka identified 10.8% of severe sepsis patients among total admission and mortality was 49.2% (Faruq et al., 2013).

The causative organism of adult sepsis has regional diversity. The increasing trend of AMR is a great concern particularly for LMIC. Bacterial infections are the commonest cause of adult sepsis in both HIC and LMIC, although non-bacterial causes are also

important for LMIC (Rudd et al., 2018, Schultz et al., 2017). Non-bacterial causes of sepsis include malaria, dengue and other viral haemorrhagic fever, and others (Rudd et al., 2018, Schultz et al., 2017). The prevalence of multi-drug resistant (MDR) bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamases (ESBL) producing bacteria, carbapenemase-producing Enterobacteriaceae (CPE) etc are higher in LMIC compared to HIC (Schultz et al., 2017, Vernet et al., 2014). A range of Gram-positive and Gram-negative bacteria cause adult sepsis globally, although the mortality burden from Gram-negative organisms is higher (Chatterjee et al., 2017, Daniels et al., 2018, Deen et al., 2012, Gotts and Matthay, 2016, Limmathurotsakul, 2017, Schultz et al., 2017). In South and South-East Asia, almost two-thirds of bacterial sepsis in adults is due to Gram-negative bacteria (Chatterjee et al., 2017, Deen et al., 2012, Limmathurotsakul, 2017). The individual burden of different Gram-negative organisms in sepsis is discussed in the next section, but *Burkholderia pseudomallei* (BP), the Gram-negative cause of melioidosis, is identified as an important cause of adult sepsis in Asia (Chaowagul et al., 1989, Deen et al., 2012, Limmathurotsakul, 2017).

1.2. Gram-negative bacteria causing sepsis

Bacterial infections are responsible for the major proportion of sepsis in the world, and amongst them Gram-negative bacteria are the main culprit (Deen et al., 2012, Limmathurotsakul, 2017). A systematic review on community-acquired bacterial bloodstream infection in South Asia identified Gram-negative bacteria as responsible for 77.4% of all positive isolates amongst adult febrile patients admitted to hospital (Deen et al., 2012). Another multicentre multinational study estimated that 60% of

bacterial sepsis cases are due to Gram-negative bacteria (Cohen, 2002). The top four causative organisms are *Salmonella enterica* serotype Typhi (*S.* Typhi; 29.6%), *Escherichia coli* (*E. coli*; 12%), *Pseudomonas aeruginosa* (*P. aeruginosa*; 10.2%) and *Klebsiella pneumoniae* (*K. pneumoniae*; 7.6%) (Deen et al., 2012).

E. coli is a classical extra-cellular, Gram-negative, oxidase-negative, motile or non-motile rod-shaped bacterium (Croxen et al., 2013, Silva, 2012). It belongs to the Enterobacteriaceae family and can grow both aerobically and anaerobically (Croxen et al., 2013). This bacterium was first isolated in 1885, and was fully characterized in 1954 (Croxen et al., 2013). It is a commensal organism for the gastrointestinal tract in humans; however, pathogenic strains cause a broad range of diseases such as urinary tract infection (UTI), bloodstream infection (sepsis), diarrhoeal illness (watery and bloody), and neonatal meningitis (Croxen et al., 2013). Important virulence factors are different classes of adhesins (fimbrial or afimbrial), Type III secretion system (T3SS), outer membrane protein, and some toxins (oligopeptide, AB, and RTX pore-forming toxins) (Mainil, 2013). The toxins can interact directly with epithelial cells of the intestinal, respiratory and urinary tracts, and facilitate invasion (Mainil, 2013). *E. coli* has the capacity to rapidly develop resistance against beta-lactam antibiotics. ESBL is now a growing concern worldwide and this results from the production of plasmid-borne beta-lactamase, especially from those belonging to the CTX-M family (Ruppé et al., 2015). ESBL-encoding plasmids often bear resistance determinants for other antibiotics such as Carbapenem and aminoglycosides.

P. aeruginosa is another classic example of extracellular Gram-negative rod-shaped bacteria, with intracellular survival capacity (Silva, 2012). It is a flagellated catalase- and oxidase-positive facultative anaerobe (Gellatly and Hancock, 2013). *P.*

aeruginosa is a metabolically versatile ubiquitous gamma-proteobacterium that survives easily in the environment, and colonizes humans, animals and plants (Klockgether and Tümmler, 2017). It is one of the commonest organisms responsible for nosocomial infection, and is responsible for considerable morbidity and mortality. *P. aeruginosa* commonly causes acute pneumonia and chronic lung infection, infection of wounds and burns, otitis externa, keratitis and sepsis (urinary tract, lung and kidney) particularly in immunocompromised subjects (Gellatly and Hancock, 2013, Klockgether and Tümmler, 2017). The major virulence factors include adhesins (flagella, type 4 pill), lipase, phospholipase, elastase, alkaline phosphatase, exotoxin-A, pyocyanin and others (Gellatly and Hancock, 2013). *P. aeruginosa* is a bacterium of major concern because of its capacity to develop rapid AMR in nosocomial infections (Gellatly and Hancock, 2013).

S. Typhi is a member of the Enterobacteriaceae family (Parry et al., 2002) and is a classical intracellular Gram-negative bacterium responsible for causing typhoid or enteric fever (Silva, 2012). People become infected through contaminated food and water, with a major disease burden in LMIC (Parry et al., 2002). The bacterium is a flagellated aerobic rod-shaped organism and is able to survive and replicate within mononuclear phagocytic cells (Johnson et al., 2018, Parry et al., 2002). A systematic review identified it as the commonest cause of bacterial blood infection in South and South-East Asia (Deen et al., 2012). Major virulence factors include typhoid toxin, Vi antigen, flagella, different effector proteins and T3SS (Johnson et al., 2018). MDR to typhoid is now a global concern (Parry et al., 2002).

K. pneumoniae is a Gram-negative encapsulated non-motile facultative anaerobic bacteria first isolated and discovered in 1882 (Ashurst J and Adam, 2018). It is commonly found in the environment and typically causes pneumonia in

immunocompromised populations such as those with diabetes or hazardous use of alcohol (Ashurst J and Adam, 2018). *K. pneumoniae* belongs to the Enterobacteriaceae family and can exhibit a high degree of virulence and AMR once it colonizes the human mucosal surface (Ashurst J and Adam, 2018). It is an extracellular pathogen with an intra-macrophage stage like *P. aeruginosa* (Belon and Blanc-Potard, 2016). The most important virulence factors are polysaccharide capsule (77 different types), lipopolysaccharide (LPS; responsible for sepsis and septic shock), fimbriae and siderophores (Ashurst J and Adam, 2018). There is much concern around growing evidence of AMR (ESBL and CPE) in *K. pneumoniae*.

1.3: Melioidosis

Melioidosis is a deadly infectious disease of humans and animals caused by the Gram-negative environmental soil and water born pathogen *Burkholderia pseudomallei* (BP) (Cheng and Currie, 2005, Currie, 2015, Wiersinga et al., 2012). Whitmore and Krishnaswami first recognised this septicaemia disease in 1911 among morphine addicts in Rangoon, Burma (now Yangon, Myanmar) (Currie, 2015, Whitmore, 1913, Whitmore and Krishnaswami, 1912). They had isolated the bacillus and noted clinical and microbiological similarity (except it was motile) to *B. mallei*, the cause of glanders (Currie, 2015). Later in 1921, Stanton and Fletcher proposed the name of melioidosis, meaning ‘resembling distemper of asses’ as originating from the Greek word ‘melis’ (Currie, 2015, Stanton and Fletcher, 1921). The genus *Burkholderia* contains over 40 species and among them other pathogenic members include *B. mallei* and *B. cepacia* (Wiersinga et al., 2012). *B. mallei* is highly infectious, virulent to humans, and causes glanders in solipeds (mammals that have a single hoof on each foot like horse, donkey, goats and others) (Wiersinga et al.,

2012, Wiersinga et al., 2018). *B. cepacia* is an important cause of opportunistic infection in patients with cystic fibrosis (Wiersinga et al., 2012). *B. thailandensis,* found in the soil in Thailand and Australia, and *B. oklahomensis*, in the Midwestern United States, are much less virulent and rarely cause disease in humans (Dance, 2000, Dance et al., 2018, Wiersinga et al., 2012). The U.S. Centre for Disease Control (CDC) recently upgraded BP to be a Tier 1 Select Agent for biodefence, based on concerns of high mortality, inhalational route of entry, diagnostic difficulty, intrinsic resistance to standard antibiotics and lack of a vaccine (Currie and Kaestli, 2016, Wiersinga et al., 2018).

1.3.1. Epidemiology

1.3.1.1. Presence in the environment

BP is a ubiquitous organism, and has been long known to be present in the soil and surface water in Southeast Asia and northern Australia (Currie and Kaestli, 2016). A recently published mathematical model predicted southeast and South Asia, tropical Australia, Western sub-Saharan Africa and South America as the highest risk zone (Figure 2), (Limmathurotsakul et al., 2016b). The bacteria is found most abundantly in soil at a depth of ≥10 cm from the surface (Limmathurotsakul et al., 2013, Wiersinga et al., 2018). The bacteria is dislodged from deeper soil to the surface during the rainy season, and can multiply (Limmathurotsakul et al., 2013). The regression model also identified a strong association between the presences of BP and high rainfall, temperature and soil type (Limmathurotsakul et al., 2016b). Anthrosol (soil modified by human activities like farming) and acrisol (clay rich soil) are both soil types that are

strongly associated with the presence of this organism (Limmathurotsakul et al., 2016b).

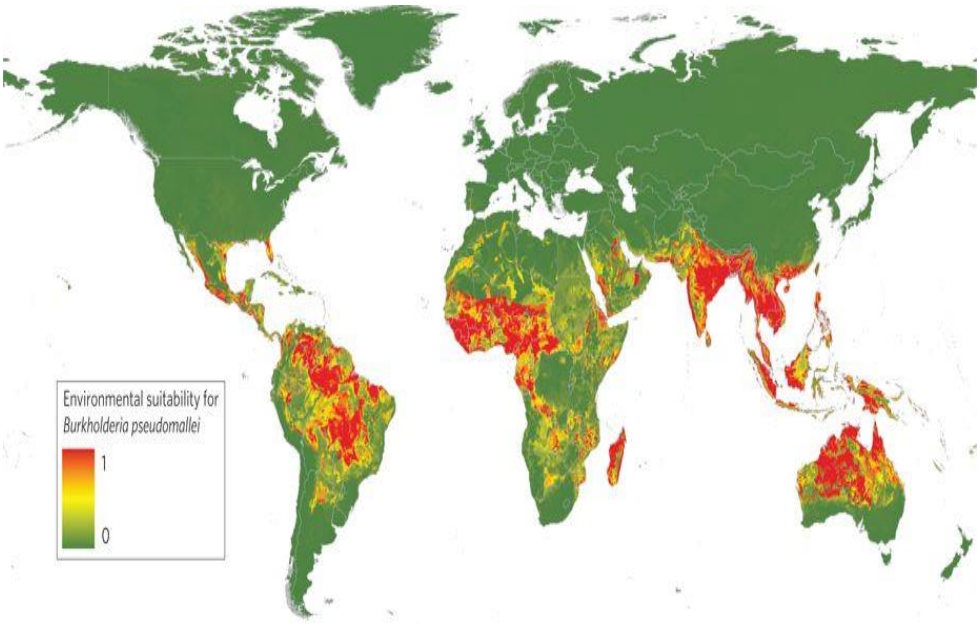

Figure 1.2: Predicted environmental suitability for *BP* persistence. Areas of high environmental suitability are shown in red and areas of low suitability in green (Limmathurotsakul et al., 2016b). Permission taken for reuse (Rights link: 4647340786826)

The presence of the bacteria in the environment was also found to be highly associated with high soil salinity and a high proportion of gravel in the soil (Limmathurotsakul et al., 2016b, Pumirat et al., 2010, Stopnisek et al., 2014). BP can survive in extreme environmental conditions, such as in nutrient-deprived water (distilled), nutrient-depleted soil or even in desert environments (Hantrakun et al., 2016, Pumpuang et al., 2011, Yip et al., 2015). In both Northern and Western Australia, melioidosis outbreaks have been linked with unchlorinated or inadequately chlorinated and contaminated water supplies (Currie et al., 2001, Inglis et al., 1999). The same issue was also found in a rural area of Thailand where drinking water was unchlorinated, and the multi-locus sequence type (MLST) of BP was matched in clinical and water samples, strongly supporting ingestion of contaminated water as a route of transmission (Limmathurotsakul et al., 2014). BP-specific T3SS ORF2 DNA level was also identified in the air of a district in southeast Taiwan where melioidosis

cases are common (Chen et al., 2015). The findings were identical with the soil, aerosol and human isolates of the corresponding endemic area (Chen et al., 2015).

1.3.1.2. Global burden of melioidosis cases

After Burma in 1911, melioidosis cases were reported from Malaysia and Singapore in 1913, from Vietnam in 1925, from Indonesia in 1929, from Sri Lanka in 1927, from the African continent in 1936 and others (Currie, 2015, Dance, 1991) and from outbreaks in South America (Rolim et al., 2018). Although the majority of documented acute melioidosis cases in the literature come from Thailand and Australia to date, it was not reported in these countries until 1955 and 1949 respectively (Currie, 2015, Dance, 1991). In 2000 it was estimated that Thailand had around 2000 to 3000 cases of melioidosis each year (Leelarasamee, 2000). The recent regression model estimated around 165,000 (95% confidence interval 68,000-412,000) human melioidosis cases in 2015 worldwide (incidence rate of 5/100,000 people at risk per year) from which 89,000 (36,000- 227,000; 54%) people died (Figure 3) (Limmathurotsakul et al., 2016b, Wiersinga et al., 2018). The model also estimated 45 severely underreported endemic countries and 34 probably endemic countries that have never reported any cases.

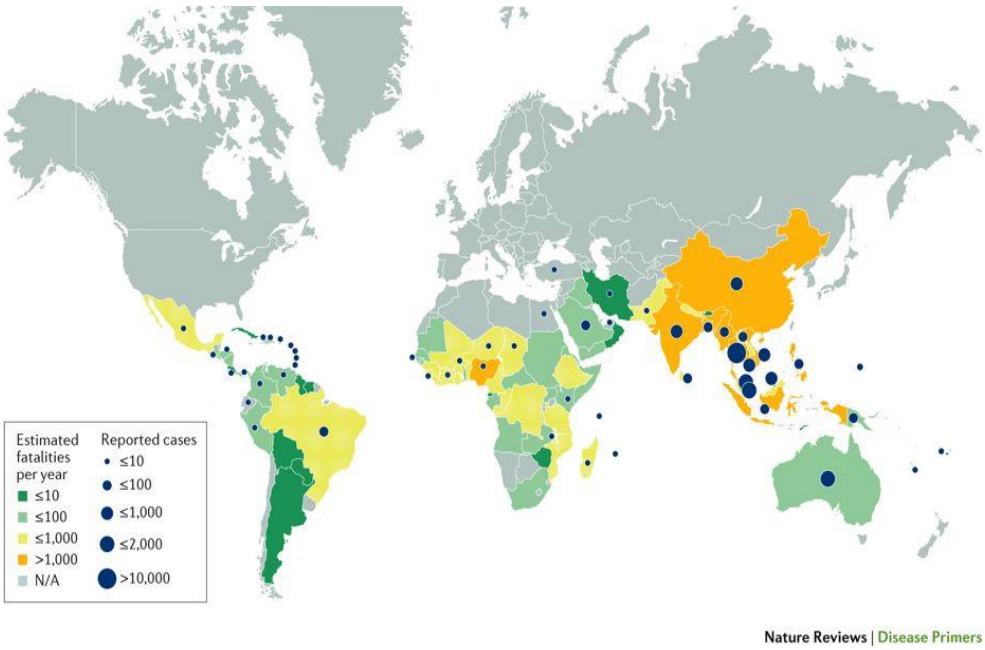

Figure 1.3: Estimated worldwide mortality and reported cases of melioidosis (Wiersinga et al., 2018) Permission taken for reuse (Rights link: 4647340442378)

Earlier studies predicted that 40% of all melioidosis cases occur in the East Asia and Pacific region, where it is reported as endemic, although this model now predicted that South Asia alone bears 44% of the overall caseload (Limmathurotsakul et al., 2016b). Due to the lack of diagnostic resources and awareness among clinicians and microbiologists, melioidosis is seriously underreported from countries like India, Bangladesh, Nigeria and other African countries. Melioidosis mostly affects middle-aged and older adults, although any age group can be affected (Currie et al., 2010). Large case series published from Australia and Thailand have found the median age of melioidosis patients to be around 50 years, but around 10% of cases in the paediatric population (Currie et al., 2010, Limmathurotsakul et al., 2010, McLeod et al., 2015).

Comparing the burden of mortality estimated by the Limmathurotsakul model with global mortality estimates for other infections in the Global Burden of Diseases 2015 study (GBD Collaborators, 2015), the annual global predicted mortality burden

[89,000] from melioidosis is similar to measles [95,600] and significantly higher than leptospirosis [50,000] and dengue infection [12,500] (Limmathurotsakul et al., 2016b). However, the World Health Organization (WHO) still does not include melioidosis in the updated neglected tropical diseases list (WHO, 2018, Wiersinga et al., 2018), perhaps due to a perceived lack of solutions to control the disease.

1.3.1.3. Burden of melioidosis in Bangladesh

Melioidosis may be a significant undiagnosed cause of infection and death in Bangladesh based on anecdotes and case reports. However, the senior internal medicine consultants in Bangladesh believe that melioidosis is not a considerable problem (personal communication). Limmathurotsakul *et al.* predicted around 16,931 cases annually with a case fatality rate of 56% (Limmathurotsakul, 2016). In a recent review, I identified 45 culture confirmed cases in 1961 to 2017 reported from Bangladesh (Chowdhury et al., 2018). Cases have so far been reported from sixteen districts out of 64, with the majority (14/39; 36%) of cases in farmers (Chowdhury et al., 2018). The most common primary clinical presentation was skin/soft tissue abscess (27%), septic arthritis (20%), pneumonia, and deep-seated abscess/organ abscess (14%). The majority of the cases were diagnosed during rainy season (June to September), and 83% of the total patients had diabetes mellitus (DM) (Chowdhury et al., 2018). The probable reason behind the mismatch of case numbers between the Limmathurotsakul prediction model and the published literature is the lack of awareness among clinicians and microbiologists, and lack of diagnostic facilities. For the last few decades majority of the melioidosis cases reported in Bangladesh have been diagnosed at only one facility, The Bangladesh Institute of Research and

Rehabilitation in Diabetes, Endocrine and Metabolic disorders (BIRDEM) Hospital microbiology laboratory.

1.3.1.4. Risk factors

One or more risk factors are present in up to 80% of adult patients with melioidosis (Currie, 2015). Children with melioidosis are less likely to have risk factors, and are more likely to have acquired disease due to a large bioburden of bacteria overcoming normally healthy host defences, due to exposures such as playing in paddy fields. DM is the most common risk factor for melioidosis, and individuals with DM have a 12-fold higher risk compared to Non-DM (Currie et al., 2004, Limmathurotsakul et al., 2010, Wiersinga et al., 2018). DM is present in more than 50% of all patients with melioidosis worldwide (Wiersinga et al., 2018). Other risk factors include hazardous alcohol use (12–39%), chronic lung disease (12–27%), chronic renal disease (10–27%), thalassemia (7%), glucocorticoid and other immunosuppressive therapy (< 5%), and cancer (< 5%) (Chaowagul et al., 1989, Currie, 2015, Currie et al., 2010, Limmathurotsakul et al., 2006, Suputtamongkol et al., 1999). People with DM represent a target population for a vaccine for melioidosis; for example, the annual incidence of melioidosis amongst adults with DM in Northeast Thailand is 145.7/100,000 (Peacock et al., 2012). The background mechanism for most of the risk factors for melioidosis are likely to be due to multi-level defects in innate, humoral and adaptive cellular immunity, which is discussed in section 1.3.3 and 4. Healthy individuals can get melioidosis if a large bioburden of infection is received, for example in adventure race athletes (Hill et al., 2013) in endemic regions or in

tsunami survivors (Chierakul et al., 2005). However, a healthy individual who has intact immune status is unlikely to die from melioidosis (Currie, 2015).

1.3.1.5. Clinical manifestations of melioidosis

The main routes of transmission of melioidosis are cutaneous inoculation and inhalation, but a case-control report from Thailand and two outbreaks in Australia also prove ingestion to be a mode of entry (Currie et al., 2001, Inglis et al., 1999, Limmathurotsakul et al., 2014). There are some rare, uncommon but documented routes of transmission such as mother-to-infant transmission through breast milk (Ralph et al., 2004), sexual transmission (Currie, 2015), zoonotic transmission from infected animals (Choy et al., 2000) and laboratory transmission to laboratory staff (Currie, 2015, Wiersinga et al., 2018). Severe weather events such as cyclones, tsunamis, typhoons, and hurricanes can shift the mode of transmission from inoculation to inhalation (Chen et al., 2014, Cheng et al., 2006a, Ko et al., 2007).

Clinical presentation and outcome depends on several factors such as the presence or absence of risk factors, route of transmission, bacterial load, virulence factors and genetic variation of strain (Currie, 2015, Wiersinga et al., 2012). The incubation period for acute melioidosis ranges from 1 to 21 days (mean 9 days) (Currie et al., 2000). Clinical manifestations vary widely from localized cutaneous infection to severe systemic infection such as sepsis and death. Every system of the body can be involved due to the haematogenous spread of the bacteria (Figure 4). Around 40-60% of patients are admitted with bacteraemia, and approximately 20% of all cases are admitted with septic shock (Currie, 2015).

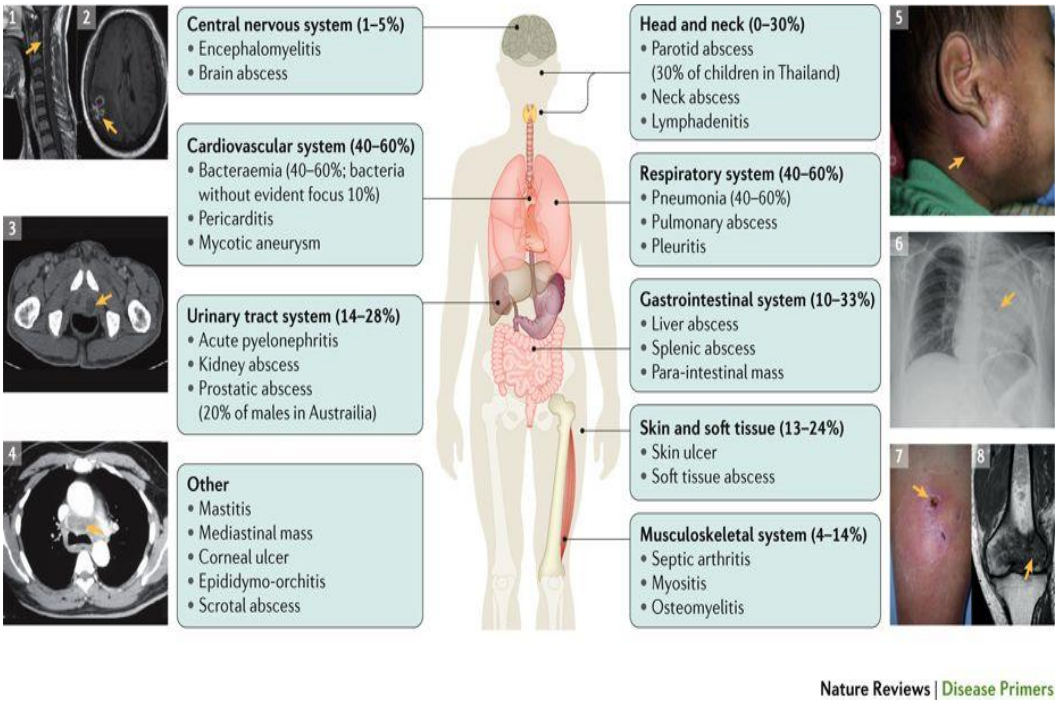

Figure 1.4: Clinical manifestations of melioidosis with the percentage of systemic involvement (Wiersinga et al., 2018). Permission taken for reuse (Rights link: 4647340442378)

Around 11% of cases may present with chronic melioidosis (presence of symptoms for more than 2 months) (Currie, 2015, Wiersinga et al., 2012). The two major chronic manifestations are non-healing skin ulcer or abscess (which fail to respond to anti-staphylococcal therapy), and pneumonia mimicking tuberculosis (hence melioidosis is also known as 'the great mimicker') (Currie, 2015). There are a few important differences in clinical presentation noted between patients in Australia and Southeast Asia. Neurologic melioidosis (in the form of brainstem encephalitis, motor weakness and cranial nerve palsies) is reported in 4% cases in Australia, whereas it is rare in Northeast Thailand (Currie, 2015, Wiersinga et al., 2018). On the other hand, suppurative parotitis is a common manifestation (up to 40%) in children of Thailand, although it is extremely rare in Australia (Wiersinga et al., 2018). These differences could possibly be explained by genetic variation of the strains (e.g. the presence of the bimABm gene that encodes neurological manifestations in strains restricted to

Australia) (Currie, 2015, Sarovich et al., 2014), genetic variation of the human host, and differences in diagnosis rates. The overall case fatality rate of melioidosis is between 10 to 50% (approximately 14% in Australia and 40% in Thailand) (Currie et al., 2010, Wiersinga et al., 2012, Wiersinga et al., 2018). Some of the difference in case fatality rate will be due to differences in clinical care (e.g. door-to-needle time for antibiotics, general supportive care for sepsis etc.)

1.3.1.6. Diagnosis of melioidosis

Microbiological culture is the gold standard for the diagnosis of melioidosis. BP is not part of the normal human flora, and therefore isolation from any clinical specimen should be considered as pathological (Wiersinga et al., 2018). The bacteria grows readily on most routine laboratory media, but it is often dismissed as contamination or not recognized, or misidentified as other bacteria such as environmental *Pseudomonas* spp. of low virulence, *Burkholderia cepacia* or *Bacillus* spp (Hoffmaster et al., 2015, Wiersinga et al., 2018). The reason behind this is the unawareness and unfamiliarity of laboratory personnel with the appearance of the bacterial colonies. Therefore, it is strongly recommended that any non–*Pseudomonas aeruginosa*, oxidase-positive, Gram-negative bacillus isolated from any clinical specimen from a patient in an endemic area should be suspected to be BP (Hoffmaster et al., 2015). In addition, based on antibiogram, any Gram-negative bacilli that are oxidase-positive, typically resistant to aminoglycosides (e.g., gentamicin), colistin, and polymyxin but sensitive to amoxicillin/clavulanic acid should be considered as BP (Hoffmaster et al., 2015). Blood culture is the most important and easily available sample, as bacteraemia in acute melioidosis is common,

although the overall sensitivity is around 60% (Hoffmaster et al., 2015). Use of specific media like Ashdown agar media can improve the sensitivity (Hoffmaster et al., 2015). Ashdown agar media is a selective media composed of trypticase peptone, 4% glycerol, 5 mg of crystal violet per litre, 50 mg of neutral red per litre, and 4 mg of gentamicin per litre (Howard and Inglis, 2003). Crystal violet and gentamicin is used to suppress the growth of other bacteria and glycerol helps the growth of BP.

A monoclonal antibody-based latex agglutination test is now in use in Thailand and some other countries with the sensitivity varies between 69.5% to 95.1% and specificity between 56.9% to 99.7% (Anuntagool et al., 2000, Duval et al., 2014, Suttisunhakul et al., 2015). The latex test could be a useful test worldwide, particularly in resource-poor settings to improve the rate of diagnosis. Melioidosis diagnostic experts are encouraging the availability and use of this tool to screen all suspected cases (Hoffmaster et al., 2015).

Throat or rectal swab culture and the centrifuged deposit from urine culture are also highly recommended in all cases of suspected melioidosis, even in the absence of relevant focal symptoms (Hoffmaster et al., 2015). Materials from other sites such as abscesses can also be cultured. Repeat culture of a suspected case whose previous culture was negative is also recommended (Wiersinga et al., 2018). A rapid diagnostic test and suitable alternatives to culture are currently research tools only. Several antigen detection assays such as immunofluorescence assay (IFA), polymerase chain reaction (PCR), and lateral flow immunoassay (LFI) are being evaluated for rapid detection of BP. Both IFA and LFI have low sensitivity, especially for blood samples (Wiersinga et al., 2018), although show some promise when tested on sputum and pus where the bacterial load is higher (Hoffmaster et al., 2015). Both

of these tests require further improvement and extensive evaluation in different real-world clinical settings. Although the sensitivity of PCR compared to a gold standard of blood culture is improving, it is expensive, can only be performed in a reference laboratory and not currently suitable for use in resource-poor settings where melioidosis in endemic (Currie, 2015, Hoffmaster et al., 2015).

Serological diagnosis is difficult and challenging because of the background high seropositivity of healthy endemic populations, and poorly characterized antigen (Wiersinga et al., 2018). The indirect haemagglutination assay (IHA) to detect antibody has been tried as a diagnostic test in endemic settings with low success due to low specificity and sensitivity (Chaichana et al., 2018). In Thailand, an immunochromogenic cassette test (ICT) and IHA were compared on two occasions, using culture as a gold standard and the sensitivity (67-82% & 73% respectively) and specificity (47-80% & 64-68% respectively) of both were low (Cheng et al., 2006c, Wuthiekanun et al., 2004). The reported sensitivity of IHA was only 56% in Australia (Cheng et al., 2006b). Promising news is the recent development of target antigen Hcp1-specific ICT (Hcp1-ICT), which showed 88.3% sensitivity and 86.1% specificity in Thai acute melioidosis patients compared to blood culture (Phokrai et al., 2018). This test has the potential as a diagnostic screening test in resource-limited countries in future. Another promising tool to improve serodiagnosis could be protein microarray (Wiersinga et al., 2018). An array containing 20 recombinant and purified BP proteins was investigated using serum from Northeast Thailand, with promising results (sensitivity 86.7% and specificity 97%) (Kohler et al., 2016). Manufacturing a low-cost version of this array for LMIC is a further challenge, but further studies of its use in the field are underway.

1.3.2. The immunopathogenesis of melioidosis

BP is a facultative intracellular organism that has the capacity to invade, survive and replicate in polymorphonuclear leucocytes, macrophages and other cell lines (Currie, 2015). The genetics and virulence of the bacterial strain BP K96243 have been described in detail (Sim et al., 2008, Wiersinga and van der Poll, 2009). It is composed of two chromosomes of 4.07 mega base pairs and 3.17 mega base pairs, of which 86% is the core genome and the rest is accessory genome, associated with virulence factors (Holden et al., 2004, Sim et al., 2008). The most notable virulence factors include LPS and other surface polysaccharides, lipid- A, peptidoglycan, flagella (type IV pili), secretion systems, and quorum sensing (Wiersinga and van der Poll, 2009). Capsular (surface) polysaccharide is important for intracellular survival of the bacteria by reducing complement factor C3b deposition (Reckseidler-Zenteno et al., 2005). Quorum sensing plays a diverse role in the context of cytotoxicity, cell invasion and AMR (Ulrich et al., 2004). T3SS and type VI secretion system (T6SS) are vital for cell invasion and intracellular survival of BP (Burtnick et al., 2011, Stevens et al., 2002). Type IV pili and flagella motility deliver a crucial role for attachment and adhesion of the bacteria with epithelial cell surface (Chuaygud et al., 2008, David et al., 2015). Other secretion systems such as type IV and type V also enhance cell adhesion and invasion (Wiersinga et al., 2018).

After invasion, BP replicates rapidly, causes cell lysis, infects the adjacent cell and in this manner can spread fast. Following endocytosis, an endocytic vesicle forms to surround the bacteria but with the help of T3SS (Figure 5), the bacteria can escape from the vesicle before degradation (Wiersinga et al., 2018). The T6SS and type 2-secretion system also play important roles in vesicle escape. In addition, a lysosomal enzyme (ecotin) homologue, resistance to oxidative stress and resistance to

antimicrobial cationic peptide help the bacteria to resist degradation during its stay inside the vesicle (Figure 5) (Ireland et al., 2014). BP also manages to escape autophagy. T3SS effector protein BopA and translocator protein BipD can block the sequestration in endocytic vesicles (Figure 5) (Gong et al., 2011). This effect ultimately prevents microtubule-associated proteins 1A/1B light chain 3B (LC3)-associated autophagy (Gong et al., 2011).

Once the bacteria reaches the host cell cytoplasm, T6 and T4 secretion systems play a crucial role in facilitating actin-mediated membranous protrusion and polymerization to spread and fuse to neighbouring cells (Kespichayawattana et al., 2000, Stevens et al., 2005). By these processes, BP forms multinuclear giant cells (like in tuberculosis), eventually dies and form plaques as well as causing damage to the host cell (Wiersinga et al., 2018). The plaques ultimately act as a reservoir for latent infection and further replication of bacteria (French et al., 2011). Bacteria have also been found to survive within the nucleus, which is another potential site for persistence and later recrudescence (Vadivelu et al., 2017). BP uses its toxin-antitoxin systems to survive under stressful conditions and enable persistence (Wiersinga et al., 2018). In addition to direct cell-to-cell spread, BP also spreads via the blood and lymphatic systems, and causes sepsis and infection to secondary sites (Wiersinga et al., 2018). BP has the capability to remain viable within dendritic cells (DC) (Williams et al., 2014). BP-infected DC can act as a vehicle to contribute to further systemic spread of infection (Williams et al., 2014).

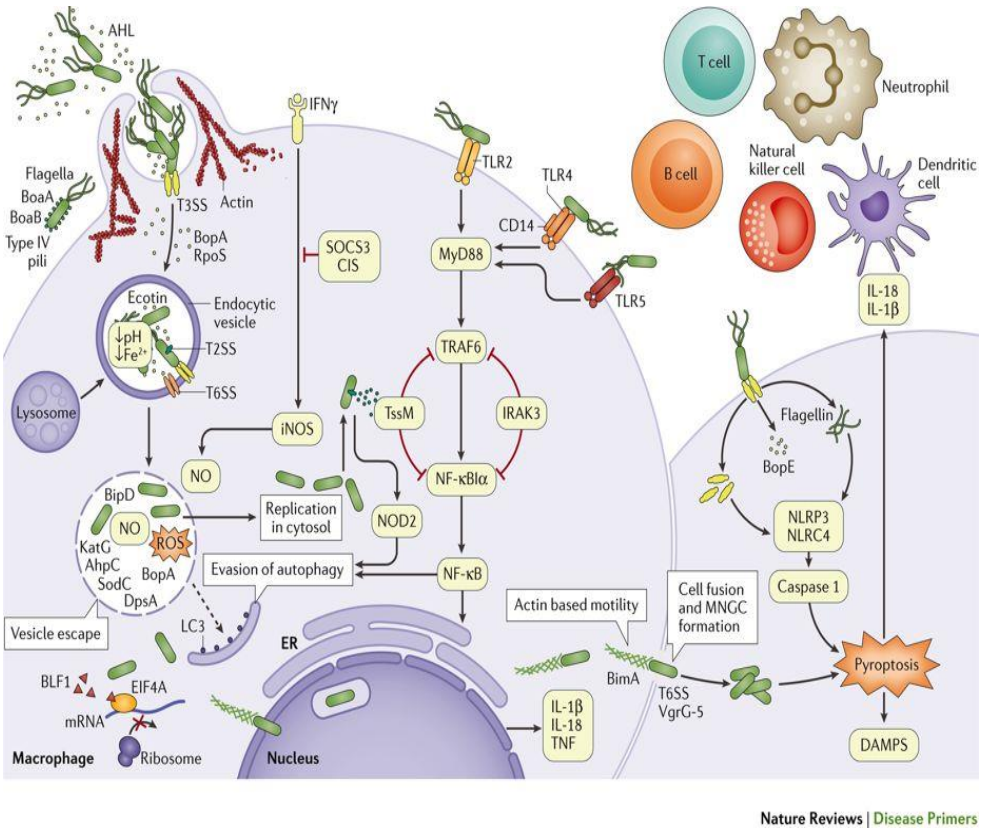

Figure 1.5: Diagram illustrating the different stages of host-pathogen interaction and immune pathogenesis of melioidosis (Wiersinga et al., 2018). Permission taken for reuse (Rights link: 4647340442378)

1.3.3. The innate immune response to BP

Immediately after epithelial cell invasion by the bacterium, the complement system is activated. However, because of the presence of outer capsular polysaccharides, the function of the host membrane attack complex is hampered and hence the bactericidal activity (Wiersinga et al., 2018). As a result, bacteria remain viable within the phagocytes and in serum (Lazar Adler et al., 2009). Various Toll-like receptors (TLRs) notably TLR2, TLR4 and other pattern recognition receptors (PRRs) like CD14 are the first to detect host invasion by BP (Figure 5) (Koh et al., 2013, Wiersinga et al., 2007a). TLRs recognize conserved pathogen-associated molecular patterns (PAMPs), and through a number of signalling proteins such as myeloid differentiation primary response gene (MyD88), the bacteria mediate an inflammatory

immune response (Figure 5). In a mouse model, it has been demonstrated that MyD88 knockout mice had markedly reduced neutrophil recruitment to the site of infection, reduced bacterial clearance capacity and diminished activation of neutrophils compared to wild-type mice (Wiersinga et al., 2008). In another *in-vitro* study, both TLR2 and TLR4 knockout mice were less responsive to BP and had worse outcome compared to wild mice (Wiersinga et al., 2007a). Studies performed in a Thai patient cohort demonstrated increased expression of CD14, TLR1, TLR2, and TLR4 on the cell surface of monocytes and granulocytes compared to healthy controls (Wiersinga et al., 2007a). This upregulation of TLRs and CD14 led to the production of inflammatory cytokines (IL-8) via nuclear factor- κB (NF-kappa B) (Figure 5) and mitogen-activated protein kinase pathway (Hii et al., 2008) required for host defence.

In the cell cytoplasm, BP goes through another process called 'pyroptosis' (Figure 5). Pyroptosis is caspase-1 dependent cell death, due to release of IL-1β and IL-18 (Wiersinga et al., 2012). In the cytoplasm, a PRR named NOD-like receptor recognizes bacterial virulence factors and triggers this process. IL-18 can induce interferon gamma (IFN-γ) production. Eventually the complement and coagulation cascades become activated, and neutrophils are recruited towards the site of infection (Wiersinga et al., 2012). Dunachie's lab in Thailand found higher neutrophil counts as an independent predictor of mortality (Jenjaroen et al., 2015), although this may partially be a marker of higher bioburden. The role of neutrophils in the immune response to BP is complex. Neutrophils are generally thought to assist antigen presentation and development of adaptive response through pro-inflammatory process (Jenjaroen et al., 2015). However, studies have found inhibition of T cell response by neutrophils in bacterial sepsis (Pillay et al., 2012), and a myeloid shift in

bone marrow production would reduce lymphocyte production. Buddhisa *et al*. demonstrated that BP-infected neutrophils could inhibit CD4+T cell proliferation and IFN-γ production via programmed cell death protein 1 [PD-1] (Buddhisa et al., 2015). In summary, uncontrolled neutrophil invasion and activation by BP could hamper generation of an optimal adaptive immune response (Jenjaroen et al., 2015).

1.3.4. The adaptive immune response

Repeated exposures to BP give rise to detectable levels of antibodies, although their protective role is not sufficient (Cheng et al., 2006b). Sero-positivity was found in healthy individuals in endemic areas, but not enough to boost adaptive immunity (Wuthiekanun et al., 2006). Detectable levels of antibodies (titre >1:10) was found in around 60% of children in endemic areas, which peaked at age 4 and then plateaued (until age 14) (Wuthiekanun et al., 2006). However a higher titre (≥1:160) was detected in only 10% of children. An IHA titre of ≥1:40 is found in 50% of melioidosis-recovered patients 1 year after infection (Chaichana et al., 2018). Cross-reactivity with other *Burkholderia* species could also be responsible for raised IHA titres. A recent study revealed no association between IHA and survival once confounders such as age, renal function, T cell response and neutrophils are adjusted (Chaichana et al., 2018). However, current studies in the Dunachie lab in Bangkok are revealing an association between BP-specific IgG2 responses and survival, as well as demonstrating correlates of infection in functional antibody studies using a newly established BP growth inhibition assay (Chaichana *et al., in preparation*).

Therefore, a strong cell-mediated immune response is believed to be vital for protection against the progression of bacterial infection (Barnes et al., 2004). After intracellular invasion with BP, this response is essential for bacterial clearance, and T cells play an important role in this action (Ramsay et al., 2002). In an outbred mouse model, resistance to acute BP infection and survival was dependent upon *in vivo* IFN-γ production (Santanirand et al., 1999). BP was found to generate a potent IFN-γ response from natural killer (NK) cells, NK T cells, conventional T cells, and other cell types within 16 hours after infection, in an IL-12- and IL-18-dependent manner in BALB/c mice (Haque et al., 2006b). The same research group also demonstrated the role of IFN-γ secreting antigen-specific CD4+ T cells in primary protection in another study (Haque et al., 2006a). CD4+ T cells are essential for B cell isotype switching and for activation of cytotoxic CD8+ T cells and phagocytes (Lazar Adler et al., 2009, Ulett et al., 1998). Another murine study exploring the protective role of IFN-γ revealed that IFN-γ knockout mice were highly susceptible to melioidosis infection, whilst lymphocyte-deficient mice showed intermediate resistance (Easton et al., 2007). The same study also found administration of anti-IFN-γ antibodies resulted in increased bacterial load and extensive infection in C57BL/6 mice, even with a dose that is usually sub-lethal (Easton et al., 2007). Further studies also confirmed the role of NK and CD8-T cell-derived IFN-γ in controlling acute BP infection in mice (Koo and Gan, 2006, Lertmemongkolchai et al., 2001). In mice, NK cells were detectable after 5 hours and IFN-γ + CD8+ T cells within 15 hours after inoculation of bacteria (Lertmemongkolchai et al., 2001). TNF-α knockout mouse also demonstrated increased susceptibility to acute BP infection (Barnes et al., 2008).

A number of studies have explored the adaptive immune response in humans after BP infection. A study performed in Thai melioidosis patients' revealed high plasma

concentrations of IFN-γ, IL-18, IL-12 and IL-15 compared to healthy controls, although the cytokine-producing cells were not characterised (Lauw et al., 1999). The same group also found elevated levels of IFN-γ inducible protein 10 (IP-10; CXCL10) and monokine induced by IFN-γ (MIG) in blood culture-positive severe melioidosis patients compared to controls (Lauw et al., 2000). Another study performed in Papua New Guinea demonstrated higher T cell proliferation levels and IFN-γ production in patients with subclinical melioidosis compared to those with clinical melioidosis (Barnes et al., 2004). Gene expression profiling of 30 acute melioidosis patients demonstrated that the host response is dominated by both type 1 and type 2 interferon responses (Koh et al., 2013), in a transcriptomic signature indistinguishable from the signature reported for TB (Berry et al., 2010). In 200 acute melioidosis cases in Thailand, T cell responses to BP correlated with survival (Jenjaroen et al., 2015). In particular, very low CD8+ T cells were seen in fatal cases, supporting the role of this cell in defence against BP. Further support for the importance of CD8+ T cell responses in host defence against BP comes from the finding of an association between the MHC Class 1 HLA alleles B*46 and C*01 and death in this same Thai cohort (Dunachie et al., 2017).

In 2015, another study found increased mRNA expression of programmed cell death protein 1 (PD-1), in the blood of acute melioidosis sepsis patients (Buddhisa et al., 2015). PD-1 is a known regulator of T-cell activation and *in vitro*, the addition of anti–PD-1 Abs was found to restore the proliferation of CD4+ T cells and IFN-γ production from infected cells (Buddhisa et al., 2015). A different research group in Thailand has demonstrated recall T cell responses in Thai people with serological evidence of BP exposure (Nithichanon et al., 2018). Studies in chronic melioidosis patients also demonstrate T cell responses to BP. A small cluster of Australian chronic melioidosis

patients (mean time after diagnosis 23 months) showed evidence of T cell specific response to BP (Ketheesan et al., 2002). Significantly higher lymphocyte proliferation and IFN-γ production were reported, along with an increase in activated CD4+ and CD8+ T cells (Ketheesan et al., 2002). These findings are further supported by another study in a large pool of seropositive and recovered Thai melioidosis patients (Tippayawat et al., 2009), which also characterized the memory T cell response and showed clear evidence of T cell priming and their ability to make IFN- γ that correlates with the titre of anti-BP antibodies in the serum.

Although the importance of T cell in acute and chronic melioidosis is evident, we know very little on the role of specific T cell subsets on the progression of disease in humans (Wiersinga et al., 2018). A recently conducted study revealed significantly reduced number of γδ T cells, CD16+ve (CD3-CD19+CD56+) and CD16-ve (CD3-CD19-CD56+) NK cells in acute patients who died, compared to those who survived (Kronsteiner et al., 2019). No differences were found in the frequency of NKT-like cells (CD3+CD56+), regulatory T cells (T-reg) and B cells among the groups.

Mucosal-associated invariant T-cell (MAIT cells) are a recently described innate-like T cell population (unconventional T cell), which has an emerging role in the human adaptive immunity in various clinical conditions including bacterial and viral infections (Wong et al., 2017), along with a role in autoimmunity and cancer immunity (Rouxel and Lehuen, 2018). The performance of MAIT cell in BP infection has never previously been evaluated, and is a focus for this thesis.

1.3.5. The inflammatory response

A dysregulated cytokine-mediated immune response also occurs in acute melioidosis, resulting in excessive inflammation with a potentially fatal outcome (Gan, 2005). Elevated levels of pro-inflammatory cytokines (such as IL-6, IL-12, IL-15, IL-18, TNF and IFN-γ) have been observed in patients with melioidosis, and IL-6 and IL-18 are independent predictors of mortality (Simpson et al., 2000, Wiersinga et al., 2007b). Anti-inflammatory cytokines (such as IL-10 and IL-4), TNF receptor type 1 and IL-1 receptor antagonist are also upregulated during septic melioidosis (Massey et al., 2014). The role of coagulation and fibrinolysis in acute melioidosis sepsis is the subject of ongoing research.

1.4: Mucosal-associated invariant T-cell (MAIT)

1.4.1. History of discovery, its receptor and antigen

There are three major types of invariant or unconventional T cells: γδ-T cells, invariant NK T (iNKT) cell and MAIT cells. In 1993, Porcelli *et al* first identified two invariant T cell receptors (TCR) in human double negative (DN; CD4-CD8-) T cells (Porcelli et al., 1993), namely the invariant TCRα chain of iNKT cells, and Vα7.2-Jα33 (Porcelli et al., 1993). This limited TCR repertoire variance gave a clue that they might be restricted to a non-classical major histocompatibility complex (MHC). Subsequently Vα7.2-Jα33 was found to be a novel population restricted by MHC class Ib molecule (Tilloy et al., 1999). Treiner *et al.* in 2003 published their landmark findings and showed that MAIT cells are selected and/or restricted by a monomorphic MHC class I-related protein (MR1) that is markedly conserved in diverse mammalian

species (Treiner et al., 2003). They also identified their abundance on the crypts and lamina propria of the gut of human and mice, and named this T cell population as ‘MAIT’ cell (Treiner et al., 2003).

MR1 is an antigen-presenting protein first described in 1995 (Hashimoto et al., 1995). A single gene outside the MHC locus on chromosome 1 encodes it. The α1-α2 domains of human and mouse MR1 are 90% identical, compared to CD1 which is around 60% identical (Riegert et al., 1998, Tsukamoto et al., 2013, Yamaguchi et al., 1998). MAIT cells are absent in MR1-negative mice (Treiner et al., 2003). Therefore, MR1 expression is essential for the development of MAIT cells (Treiner et al., 2003). MR1 is expressed ubiquitously in a similar manner to MHC class I expression (Hashimoto et al., 1995, Riegert et al., 1998), and has an antigen-presenting function. It resides in late endosomes (multivesicular), and co-localises with lysosomal-associated membrane protein and calreticulin (Huang et al., 2008). This group also revealed that MR1 surface expression and MAIT cell activation both decreased after inhibiting the acidification of the endocytic compartments (Huang et al., 2008). MR1 traffics through the endocytic compartments, and allows MAIT cells to sample antigens from both endocytosed and endogenous sources (Huang et al., 2008).

The majority of MAIT cells express the TCR α chain, Vα7.2-Jα33, Jα12 or Jα20 in humans, and Vα19-Jα33 in mice (Reantragoon et al., 2016, Reantragoon et al., 2013, Tilloy et al., 1999). A mutagenesis study demonstrated that the TCR α chain is responsible for most of the contacts with MR1 (Reantragoon et al., 2012). The conserved Tyr95α residing within the CDR3α loop is positioned directly over the ligand-binding cavity, and forms a hydrogen bond with the vitamin B metabolites bound by MR1 (Patel et al., 2013, Reantragoon et al., 2012). Hence, the CDR3α loop is crucial for MAIT cell activation. The CDR3β loop is also important for activation

when recognition of MR1 is lost due to exchange of the CD3α loop with a non-MAIT TCR (Reantragoon et al., 2012).

Two pioneer studies demonstrated that MR1-restricted MAIT cells could be activated by various species of bacteria and fungus and are important for protection against those infections (Gold et al., 2010, Le Bourhis et al., 2010). The absence of MAIT cells in germ-free mice suggested that MR1 presents a conserved microbial ligand (Le Bourhis et al., 2010). A group of Australian scientists later discovered the nature of the ligand (Kjer-Nielsen et al., 2012). Through mass spectrometry, structural biology and other techniques, the group discovered that MR1 ligands are derived from folic acid (vitamin B9) and from an intermediate in the microbial biosynthesis of riboflavin (vitamin B2) (Kjer-Nielsen et al., 2018). Two riboflavin pathway derivatives, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil abbreviated as 5-A-RU, were found to be potent MAIT cell agonists, whereas folate derivative 6-formylpterin generally inhibits its activation (Kjer-Nielsen et al., 2018). 5-A-RU cannot directly activate MAIT cell, rather they do it via the generation of two unstable riboflavin intermediates 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), respectively (Corbett et al., 2014, Kjer-Nielsen et al., 2018). MR1 tetramers to these two intermediates were subsequently developed for research (Kjer-Nielsen et al., 2018). Therefore organisms which are known to possess riboflavin precursors in their synthesis pathway (including *Mycobacterium*, *Enterobacter*, *Pseudomonas*, *Salmonella*, *Escherichia coli* and various *Candida* species) can activate MAIT cells (Gold et al., 2010, Kjer-Nielsen et al., 2018, Le Bourhis et al., 2010). BP and *Klebsiella pneumoniae* also synthesize riboflavin during metabolism (Gold et al., 2010).

1.4.2. MAIT cell development

MAIT cells develop through a multistage process, starting with intrathymic selection and ultimately leading to peripheral expansion (Martin et al., 2009). MAIT cells are deduced to be selected in the thymus due to their presence in the human thymus (Martin et al., 2009), in foetal thymic organ culture, and in double iTCR alpha and TCR beta transgenic RAG knockout mice (Koay et al., 2018, Martin et al., 2009). This selection occurs by MR1-expressing double positive (DP) cortical thymocytes (Koay et al., 2016). Koay *et al*. first described the three-staged intrathymic pathway for MAIT cell development (Koay et al., 2016). Stages 1 and 2 take place within the thymus, whilst Stage 3 gradually progresses to outside of the thymus (periphery). At the first stage of development, the cells are a CD24+CD44- population in the mouse thymus and as a CD27-CD161- population in the human thymus (Koay et al., 2016). Subsequently in Stage 2, they become CD24-CD44- in the mouse and CD27+CD161- in humans. At this stage, MAIT cells require promyelocytic leukaemia zinc finger protein (PLZF) as a mediator to transit into Stage 3 (Koay et al., 2016, Martin et al., 2009). Murine experiments identified other crucial mediators such as microRNAs, IL-18 and commensal microbiota (Koay et al., 2016). Germ-free mice, which lack these mediators, showed reduced MAIT cell development and stunted cell number (Koay et al., 2016). PLZF and microbial colonization both are crucial in mice for expansion of MAIT cell in the periphery (Koay et al., 2016, Le Bourhis et al., 2010).

PLZF is also essential for Stage 3 MAIT cell development, to drive innate-like effector function in the thymus, which is followed by secretion of IFN-γ and tumour necrosis factor alpha (TNF-α) upon activation (Dias et al., 2017). This function is further

enhanced after peripheral expansion along with an increased level of PLZF expression (Dias et al., 2017). In all three stages of development, MR1 expression (as a checkpoint regulator) is crucial (Koay et al., 2016). Mature MAIT cells in Stage 3 express CD24-CD44+ in mice and CD27+/- and CD161+ in humans (Koay et al., 2016). MAIT cells exit the thymus as naïve cells, and their maturation and expansion continue in the periphery. B cells also appear to be important for peripheral expansion of MAIT cells, as found by the absence of mature MAIT cell in B cell-deficient mice (Martin et al., 2009). MAIT cell regulation is dynamic in humans, with differences in the use of co-receptors by MAIT cells between the thymus and in the periphery (Koay et al., 2018). CD4+ or DP MAIT cells are prevalent in thymus, whereas CD8+ and DP are predominant in adult blood (Koay et al., 2018). Exiting from the thymus, MAIT cells gradually acquire a memory phenotype and CD45RO expression, alongside innate-like properties with CD161 & IL-18R expression (Koay et al., 2018), as demonstrated in humans through ontogenic studies in cord blood, young and adult blood (Ben Youssef et al., 2018).

1.4.3. MAIT cell distribution

MAIT cells are more abundant in humans than in mice. MR1 tetramer studies have confirmed MAIT cell enrichment in the gut (Kurioka et al., 2016). Different anatomical sites in the gastrointestinal tract have different frequencies of MAIT cell (Figure 6). In the jejunum, around 60% of T cells have been described as MAIT cells – although in a single report - whereas the frequency is much less in the colon (10% of T cells), healthy ileum (1.5% of T cells) and rectum (2% of T cells) (Kurioka et al., 2016, Reantragoon et al., 2013). MAIT cell frequency in the liver is around 20-50% of T cells, and in the lung is around 2-4% of T cells (Kurioka et al., 2016). In the peripheral

blood, MAIT cells comprise up to 10% of circulating T cells (Dusseaux et al., 2011). Recent studies also reported the presence of MAIT cells in endometrium, cervix, kidney, prostate and ovaries (Kurioka et al., 2016). Interestingly, MAIT cells are very rare in lymphoid tissue, and they preferentially home to peripheral tissue (Dusseaux et al., 2011). This is supported by MAIT cell expression of gut-homing integrin α4β7, as well as chemokine receptors CCR6, CCR9 and CXCR6, which are primarily involved in trafficking to peripheral tissues such as liver and intestine (Dusseaux et al., 2011, Kurioka et al., 2016).

In contrast to humans, MAIT cells are rare in wild-type laboratory mice (Tilloy et al., 1999). Therefore, the majority of murine studies on MAIT cells have used invariant Vα19-Jα33 TCR transgenic mice (Kawachi et al., 2006). It has been reported that infection with *Francisella tularensis* live-vaccine strain (Meierovics et al., 2013) or *Salmonella typhimurium* (Chen et al., 2016) markedly increases the number of MAIT cells in the murine model. Characterization of wild-type murine MAIT cells using tetramers in C57BL/6 mice revealed that MAIT cells are present in the lung (3.3% of T cells), liver (0.6% of T cells) and in lamina propria (0.7% of T cells) (Kurioka et al., 2016, Rahimpour et al., 2015).

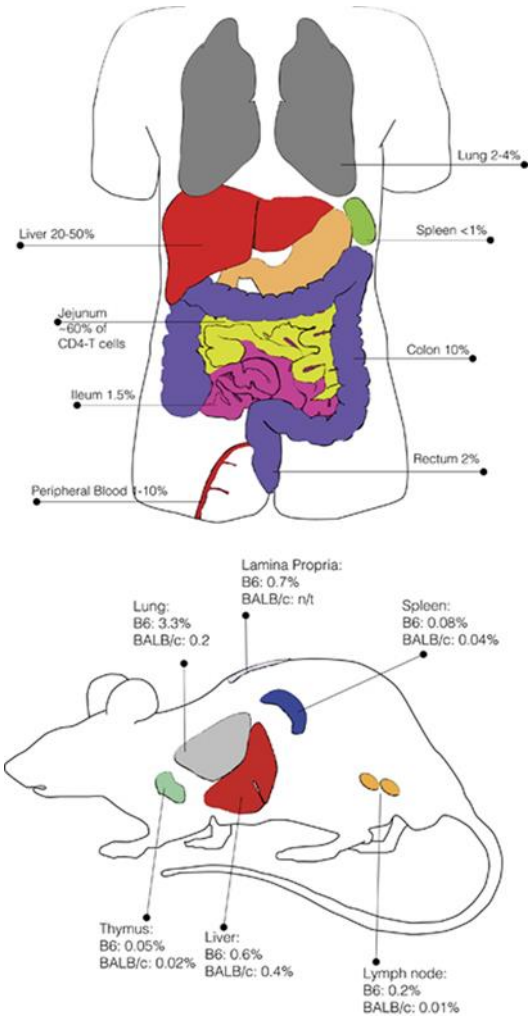

Figure 1.6: Distribution of human and murine MAIT cells in tissues. The frequency of MAIT cells is defined either by MR1 tetramers or as $CD161^{++}$ $V\alpha7.2^{+}$ within T cells (Kurioka et al., 2016).(permitted under CC BY-NC-ND 4.0 license)

1.4.4. MAIT cell phenotype and effector functions

MAIT cells were initially identified in the double negative (DN; CD8-CD4-) T cell population (Porcelli et al., 1993). However, it is now evident that the majority of MAIT cells in humans are CD8+, with a comparatively smaller population of DN MAIT cells, and a minor population of CD4+ T cells (Kurioka et al., 2017, Reantragoon et al., 2013). A distinguishing feature of MAIT cells is that the majority of CD8+ MAIT cell express the CD8αα homodimer, whereas conventional CD8+ T cells express the CD8αβ heterodimer (Kurioka et al., 2016). Another feature of MAIT cells is the high surface expression of CD161 and IL-18R (Kurioka et al., 2016). CD161 is considered

to be one of the earliest markers expressed on MAIT cells, and can be used as a surrogate marker for identification in addition to Vα7.2 (Kurioka et al., 2016).

Activation of MAIT cells occurs by two pathways: TCR dependent or independent (cytokine dependent). MAIT cells are restricted to the evolutionary conserved MR1 protein and riboflavin metabolites rRL-6-CH2OH that is able to stabilize MR1 and activate MAIT cells (Kjer-Nielsen et al., 2018, Kjer-Nielsen et al., 2012). Therefore, bacteria, which synthesise riboflavin during metabolism, activate MAIT cells via TCR (MR1) dependent pathway. On the other hand, high levels of IL-18R expression enable MAIT cells to secrete IFN-γ and TNFα in response to innate cytokines such as IL-12 and IL-18 (Kurioka et al., 2016). Upon activation, MAIT cells also release the same cytokines in an IL-12- and IL-18-dependent manner particularly in response to bacteria such as *Enterococcus faecalis*, which lack the riboflavin synthesis pathway (Ussher et al., 2014a). Viral infections such as dengue, influenza, and hepatitis C can also exhibit this functional response in a cytokine-dependent manner and are the main IL-17 producing T cells in the liver (Kurioka et al., 2016) and in adipose tissue (AT) (Magalhaes et al., 2015b). The effector function of MAIT cells also includes the secretion of the cytotoxic molecules Granzyme B (GzmB) and perforin upon activation in both MR1-dependent and independent manner (Kurioka et al., 2015). GzmB can therefore be an activation marker of MAIT cells. The proliferative response of MAIT cells is not yet well understood. Ki-67 is a protein and present during all active phases of the cell cycle (G (1), S, G (2), and mitosis, but is absent during resting stage G (0) (Scholzen and Gerdes, 2000). Therefore, its expression in human is specifically associated with cell proliferation. Adult MAIT cells lack Ki67 expression in the periphery, and proliferate poorly in mice (Dusseaux et al., 2011). MAIT cells are thought to expand in the periphery in response to bacterial infection, as supported by an increase of their frequency in the lung following *F. tularensis*

infection in wild type mice (Meierovics et al., 2013). This finding requires confirmation in humans.

1.4.5. The role of MAIT cells in health and disease

The role of MAIT cells in bacterial infections and sepsis will be discussed in Chapter 6. The frequency of MAIT cells changes in a number of viral infections in both mice and humans. It is now established that MAIT cells respond to viral infections in an MR1-independent manner (Ussher et al., 2018). Initial studies did not find evidence to support viral activation of murine and human MAIT cells. Studies of encephalomyocarditis virus, Sendai virus, Newcastle disease virus, herpes simplex virus and parainfluenza 3 virus in mice showed no evidence of activation (Le Bourhis et al., 2010). However, subsequent studies have convincingly proven that MAIT cell frequency, functionality and activation is altered by viral infections in human, as follows.

In chronic hepatitis C virus (HCV) infected patients, reduced numbers of MAIT cells were seen in both blood and liver (Beudeker et al., 2018, Bolte et al., 2017). An inverse relationship was found between the degree of inflammation, fibrosis, and MAIT cell frequency (Beudeker et al., 2018, Bolte et al., 2017). Markers of exhaustion (PD-1, TIM-3 and CTLA-4) and activation (CD38 and HLA-DR) were also found to be high in chronic HCV patients (Barathan et al., 2016). The same picture was also seen in HIV infection in both adults and children, and the functionality (as defined by MAIT production of IFN-γ) did not recover in adults after anti-retroviral therapy (Fernandez et al., 2015, Leeansyah et al., 2013, Ussher et al., 2015). MAIT cells were also depleted in the periphery in the case of HIV-HCV co-infection (Beudeker et al., 2018). Two separate studies in acute dengue and influenza (pandemic H1N1 and H7N9)

patients demonstrated that both of these viruses activate MAIT cells to induce IFN-γ through the cytokine-dependent pathway (Loh et al., 2016, van Wilgenburg et al., 2016). Upregulation of CD69 and GzmB expression was noted in both cases (Loh et al., 2016, van Wilgenburg et al., 2016). Functional depletion of MAIT cells is also seen in the blood of human T lymphotropic virus type 1 (HTLV-1) and zika virus infection (Paquin-Proulx et al., 2017).

A number of studies have also examined the role of MAIT cells in autoimmune conditions. The abundance of this cell in the gut enticed researchers to explore its role in inflammatory bowel disease. The frequency of MAIT cells was found to be reduced in the blood of both ulcerative colitis and Crohn's disease patients compared to healthy controls (Hiejima et al., 2015). Longitudinal patient studies have demonstrated that accumulation of MAIT cells at the site of inflammation is associated with improvement of colitis (Reantragoon et al., 2016). MAIT cell frequency is stunted in adult coeliac disease patients, but not in the paediatric patient population (Dunne et al., 2013). Both in severe asthma and chronic obstructive pulmonary disease (COPD), a strikingly reduced number of MAIT cells have been observed in CD8+ and DN compartment compared to healthy individuals (Reantragoon et al., 2016).

A recent review looked at the role of MAIT cells in other immune-mediated diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, psoriasis, Sjögren syndrome, long-standing type 1 DM, type 2 DM, non-alcoholic fatty liver disease and obesity (Rouxel and Lehuen, 2018). The conclusion was that MAIT cell frequencies are reduced in the blood in these conditions compared to controls, and there is typically an increase in MAIT cell frequency at the site of disease (inflamed tissue or AT), with higher expression of activation and exhaustion markers (Rouxel and Lehuen, 2018). This is compatible with migration of MAIT cells from the periphery to

sites of disease, but whether MAIT cells act as “friend or foe” in the pathogenesis remains to be elucidated. MAIT cells may show a similar pattern of migration in multiple sclerosis (MS) (Reantragoon et al., 2016).

The role of MAIT cells in malignant diseases remains to be clearly established. In cervical cancer (n=47) MAIT cells were found in a significantly reduced amount (0.987% vs. 4.008%, $p < 0.0001$) in the peripheral blood compared to healthy control (n=39) (Huang et al., 2019). Peterfalvi *et al.* reported increased MAIT cell transcripts (Vα7.2-Jα33) in solid kidney and brain tumour tissue compared with blood and control tissue (Peterfalvi et al., 2008), and studies in colorectal carcinoma and colonic adenocarcinoma (Kurioka et al., 2016) support this finding. MAIT cells can secrete IFN-γ, IL-17 and promote tumour-specific T-cell response during malignant disease (Kurioka et al., 2016), but further exploration of the specific physiological role of MAIT cells in cancer is needed.

1.5. Immune pathogenesis of Gram-negative sepsis

LPS, also known as endotoxin, forms the outer membrane of all Gram-negative bacteria (Cohen, 2002). The lipid A portion enables LPS to be embedded in the bacterial wall, and the truncated cone shape of this structure imparts maximum ability to activate the host cell membrane (Cohen, 2002). LPS is sensed via an LPS-binding protein (LBP)–LPS complex, and this forms a further complex with CD14 (an opsonic receptor) (Cohen, 2002). This complex then activates signalling through TLR. Cells which are CD14 negative such as dendritic cells, vascular endothelium and others are still able to respond to LPS in reciprocity with soluble CD14, and in acute Gram-negative sepsis patients soluble CD14 levels are high compared to healthy individuals (Landmann et al., 1995). So far, a family of 10 TLRs have been

discovered, and TLR-4 is the LPS receptor for Gram-negative bacteria (Cohen, 2002). A cell surface molecule MD-2 is required for activation of TLR4 (Cohen, 2002). Several other protein receptors such as IL-1, NF-κB, TREM-1, MDL-1, NOD1 & 2 are also important for host signalling to activate immune responses (Cohen, 2002).

Following the host-microbial interaction, a group of pro-inflammatory cytokines are released from mononuclear cells such as TNF, interleukin-1β (IL-1β), IL-12 and IL-18 (van der Poll et al., 2017). Blocking or eliminating these cytokines was found to be protective in acute sepsis animal models (van der Poll et al., 2017). Pro-inflammatory cytokines initialize secondary responses such as IL-18 inducing production of IFN-γ, which has a protective role in sepsis (Cohen, 2002). This inflammatory response triggers activation of the complement and coagulation cascades in the vascular endothelium, which are considered hallmarks of sepsis and likely to be responsible for the cardiovascular and multi-organ collapse observed in sepsis patients (van der Poll et al., 2017). Complement activation results in secretion of C3a and C5a, which hold a potent pro-inflammatory effect, activating platelets, endothelial cells and leukocytes (van der Poll et al., 2017). These tissue factors initiate proteolytic cascades, which ultimately produce fibrin from fibrinogen, block the microcirculation, and cause tissue hypoperfusion and multi-organ failure (Cohen, 2002).

These pro-inflammatory events lead to programmed cell death or apoptosis in acute infection, a key feature of sepsis (van der Poll et al., 2017). Apoptosis is responsible for severe depletion of CD4+ and CD8+ T cells, B cells and DCs in acute sepsis (van der Poll et al., 2017). CD4+ T helper 1 (TH1) cells, TH2 cells and TH17 cells are also stunted in acute bacterial sepsis patients (van der Poll et al., 2017). T cells from the spleen produce significantly lower amounts of IFN-γ and higher expression of exhaustion markers in acute bacterial sepsis compared to non-infectious cases (Boomer et al., 2011). The marker of apoptosis PD-1 was also found to be highly

expressed (in CD4 T cells) in acute sepsis patients compared to controls (van der Poll et al., 2017). Monocyte PDL1 expression can be an independent predictor of short-term mortality (28-days) in septic shock patients and may serve as a biomarker for the selection of patients for anti-PD1 or anti-PDL1 therapies (Shao et al., 2016, van der Poll et al., 2017). Cellular metabolism is another interesting mechanism that regulates inflammatory response in Gram-negative sepsis. LPS in the outer membrane of Gram-negative bacteria prompts a classical Warburg effect (a shift from oxidative phosphorylation to glycolysis) in healthy monocytes, which is an important component of initial activation of host defence (Cheng et al., 2016, van der Poll et al., 2017). This effect rapidly provides ATP and metabolic intermediates for the biosynthesis of immune and inflammatory proteins (van der Poll et al., 2017). In contrast, the leucocytes of acute sepsis patients show a generalized immunometabolic defect, which could potentially be manipulated by immune therapies (Cheng et al., 2016).

1.6. DM and Gram-negative bacterial infection

Patients with diabetes are at increased risk of infections such as upper respiratory tract infections, bronchitis, influenza-like illness, pneumonia, urinary tract infection, cellulitis, superficial and deep fungal infections and others, and have worse outcomes (Hine et al., 2017). Several studies have now confirmed a direct relationship between DM with tuberculosis. Individuals with DM are two to three times more prone to tuberculosis, and have an increased risk of treatment failure and death (Al-Rifai et al., 2017, Workneh et al., 2016).

Several published studies have examined the relation between DM and Gram-negative bacterial infections. Patients with DM have a twelve-fold increased chance

of getting melioidosis (Currie et al., 2004, Limmathurotsakul et al., 2010). A prospective longitudinal study of 241 people with DM in U.S found a significantly increased chance ($p< 0.001$) of getting *E. coli* infection compared to healthy controls (Rayfield et al., 1982). A retrospective study performed in Taiwan showed that 34% of adults (>50 years age) with *Salmonella* infections had DM (Yang et al., 1996). In a US hospital outbreak of *S. enteritidis,* people with DM had a threefold increased risk of getting an infection (Telzak et al., 1991). A meta-analysis of pyogenic liver abscess cases in China revealed that patients with DM had an increased risk of abscess with *Klebsiella spp* and *Escherichia spp* (Luo et al., 2016). Another similar study performed in Taiwan concluded that abscesses caused by *K. pneumoniae* (70.2% of the all) were more strongly associated with DM or impaired fasting glucose than liver abscesses caused by non-*K. pneumoniae* (32.5%) (Yang et al., 2004).

The mechanism behind the increased susceptibility of people with DM acquiring Gram-negative infection is probably multifactorial and related to the dysregulation of the immune system (Dunachie and Chamnan, 2018). Altered gut microbiota in DM secrete different immuno-modulatory molecules, modulate PRR pathways in the gut and ultimately alter the innate and adaptive response, making the individual more vulnerable to infection (Hooper et al., 2012). Hyperglycaemia impairs neutrophil degranulation, complement activation and phagocytosis (Hulme et al., 2017). Type 1 fimbriae are the most important virulence factor (through adhesion and colonization) for causing UTI by *E. coli* (Taganna et al., 2011). Increase fimbria adhesion has been observed in women with DM compared to non-DM (Taganna et al., 2011). The adaptive and innate immune response play a pivotal role in host defence against intracellular bacterial infection via NK cell, CD4+ T cells and CD8+ T cells (Hodgson et al., 2015). Cellular immunity is altered in DM (Hodgson et al., 2015), with lower antigen-specific IFN-γ T cell responses during acute melioidosis seen in people with

DM compared to those without DM (Jenjaroen et al 2015). Overall, DM impairs the immune system at the molecular, cellular and systemic level (Dunachie and Chamnan, 2018). Further background mechanisms of the relationship between DM and melioidosis will be discussed in Chapter 5.

2. Methods

2.1. Is melioidosis a significant cause of febrile illness in Dhaka, Bangladesh?

2.1.1. Study setting

The study was a prospective observational clinical cohort study of adults admitted with fever. The study was conducted at Dhaka Medical College Hospital (DMCH), Dhaka, which is the largest tertiary care hospital in Bangladesh comprising 2300 beds, and at BIRDEM Hospital, which has 600 beds. Patients were enrolled from Internal Medicine, Critical Care Medicine, Surgery and Orthopaedics Departments. The principal investigator (PI) of the study was Dr Fazle Rabbi Chowdhury who wrote the study protocol and patient information sheet (PIS), obtain ethics permission and oversaw the operation of the study, with support from his supervisor Prof Susanna Dunachie. Dr Chowdhury spent 6 months in Dhaka during the study in 2017. DMC is a public (government) hospital and BIRDEM is a private hospital in Dhaka. Both hospitals receive patients from all over the country as part of referral, and are therefore considered tertiary referral hospitals. Patients are usually admitted free or with a very minimum cost (admission and management costs are much less in DMC compared to BIRDEM). BIRDEM receives regular annual subsidy and funds from the ministry of social welfare, Bangladesh and other sources. Baseline investigations are also performed at a government-subsidised rate, although in the majority of cases patients (or their families) need to pay in full for blood and other cultures. In DMC, patients with any illness are admitted to a full range of specialities, whilst BIRDEM is a specialised hospital for patients with DM, other endocrine or metabolic disorders, and the majority of patients have DM.

2.1.2. Study set-up

The initial steps to set up this study were challenging. We were unable to raise a small grant to support the study, so the study was funded by the small bench fees for Dr Chowdhury's Commonwealth PhD scholarship, UK, and benefitted from ongoing research exploring the T cell response to BP for the Wellcome Trust Intermediate Clinical Fellowship grant of Prof Dunachie. The mechanism of money transfer from UK to Bangladesh is very complicated, and we were unable to transfer money directly from University of Oxford to DMC or BIRDEM to finance the work due to a lack of audit processes to satisfy the University of Oxford's monetary regulations. We therefore took the support of Mahidol-Oxford Tropical Medicine Research Unit (MORU), which is based in Bangkok, Thailand but has a longstanding collaboration with Chittagong Medical College (Eastern Bangladesh) and has an established mechanism with audit trail in place for research grant transfer to Chittagong from MORU in accordance with the University of Oxford's regulations. Another important challenge was the export of clinical samples (PBMC, plasma and serum) from BIRDEM, Dhaka to Oxford, as this required permission from multiple levels including the Ministry of Health and the Family Welfare and Ministry of Commerce, Government of Bangladesh. It took more than six months to secure the export permission after pursuing the ministries on numerous occasions, but now that is in place, to our knowledge Dr Chowdhury is the only person in Bangladesh to hold such permission, which is enduring for future research studies.

2.1.3. Ethical permissions

Written approval was obtained from the Research Ethics Committee (REC) of DMC (MEU-DMC/ECC/2015/97), BIRDEM (BADAS-ERC/EC/17/0127) and Oxford Tropical Medicine Research Ethics Committee (OXTREC: 51-16) before commencement of the study. The permission letters are in the appendix of this thesis (Annex 1-3). The informed consent form (Annex 4), PIS (Annex 5), case record form (CRF; Annex 6) and advertising material (all of which were prepared by Dr Chowdhury along with the study protocol) were also approved by the RECs. The study possessed minimal potential for harm to the environment. Prof Dunachie and Dr Chowdhury completed training in Good Medical Practice (GMP) prior to the start of the study.

2.1.4. Microbiology laboratory

The laboratory tests were performed at the Microbiology Department of BIRDEM Hospital as the best available laboratory in Bangladesh for microbiology diagnostic culture. The laboratory receives around 600-700 haemoculture bottles from all over the country each year, and patients need to pay to get the report. The department has a laboratory with two-biosafety cabinet, other essential equipment for microbiological culture, and experienced microbiologists to detect BP. The method used in BIRDEM laboratory for isolation and identification of bacteria is based on the standard microbiology procedure of Mackie and McCartney's practical microbiology (Collee, 1996). Antibiotic susceptibility testing was performed by Kirby-Bauer disc diffusion method using Clinical and Laboratory Standards Institute (CLSI), USA, 2017 guidelines. The American Type Culture Collection (ATCC) control organism strain was

used for quality assurance. The laboratory did not have any external quality assurance mechanism. However, we arranged and financed a visit from renowned clinical microbiologist, Prof David Dance from Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (LOMWRU) before commencing the study. Prof Dance went through the criteria for diagnosis and antimicrobial susceptibility test of BP with the microbiology staff. My supervisor Prof Dunachie from Oxford also visited the study sites during the project.

2.1.5. Study population

Potential study patients were any adult patient of 18 years or more who was admitted to either DMCH or BIRDEM hospital and with history of fever ≥38 degrees for more than 48 hours. We recruited 201 patients for the study during the peak rainy season between mid-July to mid-October 2017. We also recruited 25 age-matched healthy controls from the Department of Blood Transfusion, DMCH and 25 critically ill non-infectious cases to compare the specificity of immune responses with infectious patients. The diagnoses for these critical illness non-infectious cases were acute pesticide poisoning (n=6), myocardial infarction (n=4), diabetic ketoacidosis (n=1), acute chronic obstructive pulmonary disease (COPD) (n=3), snakebite (n=1) and acute stroke (n=9).

Inclusion Criteria for febrile patients

- Age ≥ 18 years old on the day of enrolment into the study
- History of fever ≥ 38 degrees for more than 48 hours
- Required hospitalization as decided by the attending physician

- Written Informed Consent has been obtained

Exclusion Criteria for febrile patients

- Admitted to the study site hospital for more than 48 hours before enrolment
- Pregnancy
- Receiving steroid therapy for more than 8 weeks
- Receiving treatment for *Mycobacterium Tuberculosis* (TB)

Inclusion Criteria for Healthy controls

- Age ≥ 18 years old on the day of enrolment into the study
- Written Informed Consent has been obtained

Exclusion Criteria for Healthy control

- Current illness requiring medical care (only for healthy control)
- Pregnancy
- Receiving steroid therapy for more than 8 weeks
- Receiving treatment for TB

Inclusion Criteria for Patient controls

- Age ≥ 18 years old on the day of enrolment into the study
- Written Informed Consent has been obtained
- Critically ill non-infectious case diagnosed by clinical features and available laboratory reports (for patients control only).

Exclusion Criteria for Patient control

- Current illness requiring medical care (only for healthy control)
- Pregnancy
- Receiving steroid therapy for more than 8 weeks
- Receiving treatment for TB

2.1.6. Sample size calculation

This was a pilot study to identify whether Dhaka is a hotspot for melioidosis. A sample size of 58 has 80% power to detect an incidence of disease of 5%, and a sample size of 200 has 80% power to detect an incidence of disease of 1%, with a two-tailed significance level (alpha) of 0.05 (Fosgate, 2009). By way of example, if our study found zero cases out of 200 febrile admissions have melioidosis, we could report that melioidosis was the cause of 0% (95% CI 0-1.8%) of febrile admissions to these two hospitals, and concluded it was not a significant health problem in Dhaka. If however we detected 10 cases of melioidosis in 200 febrile admissions, we could report that melioidosis was the cause of 5% (95% CI 2.4-9.0%) of febrile admissions, which would be considered a significant burden in Dhaka.

2.1.7. Recruitment

The resident physician (RP) or resident surgeon (RS) reviewed all the patients at DMCH and BIRDEM. The RP/RS decided which febrile patients required admission. During out-of-office hours, patients were admitted through the Emergency Department, and the emergency medical officer (EMO) reviewed the patients and decided when to hospitalize. Each day, under Dr Chowdhury's supervision, the study research assistants (RA) reviewed the record of admissions for the past 24 hours, and discussed with the RP/RS/EMO to identify patients meeting the inclusion criteria for enrolment into the study. Our RA also approached blood donors at the department of blood transfusion, DMCH to enrol the healthy controls.

2.1.8. Screening and Eligibility Assessment

The RA approached every patient identified as meeting the inclusion criteria for enrolment into the study. The screening and enrolment was performed within 24 hours. No investigation was performed as part of screening. Enrolment was as per clinical features set in the inclusion criteria.

2.1.9. Informed Consent

Prior written informed consent (Annex 4) was sought from every patient or from their attendants. The consent form and PIS (Annex 5) were in Bangla language, and outlined the exact nature of the study; what was involved for the participant; the implications and constraints of the protocol; and the known side effects and any risks involved in taking part. If the patient or their attendants were unable to read, the PIS was read to them. It was clearly stated that the participant was free to withdraw from the study with no obligation to give the reason for withdrawal. The RA discussed the study with each potential participant, answered questions and was responsible for recording written informed consent.

2.1.10. Study visit

Following obtaining written informed consent, subjects were enrolled into the study. A CRF was completed documenting the patient's demographic and clinical parameters (Annex 6). As part of routine clinical care, blood for culture was obtained. If clinically indicated, other samples such as urine, throat swab, sputum, pus, other fluids, tracheal aspirate, broncho-alveolar lavage and cerebrospinal fluid (CSF) for bacterial culture

were collected. The RA was responsible for collecting the blood, and had coordinated the collection of other samples from the enrolled patients. Adequate training was provided by the PI to re-enforce sterile and safe blood collection techniques. The RA was responsible for transport of clinical samples to the BIRDEM laboratory and provided the report to the patients, either by hand or by post. Microbiology culture of blood to look for bacteria was performed free of charge (usually patients need to pay for this). In addition, a screening test for DM (glycated hemoglobin, HbA1C) was performed free of charge and a paper copy of the report was given to each participant. The RA also communicated the results of bacterial culture and HbA1C to the relevant clinical team for each patient. A total of 15 ml blood (10 ml for culture, 3 ml for serum and 2ml for HbA1C) was collected on the first occasion.

For patients who had Gram-negative bacteria isolated on blood culture, another set of blood (15 ml) was collected and sent to the laboratory for peripheral blood mononuclear cell (PBMC), plasma and serum isolation. 20 ml blood was collected for PBMC, plasma, serum and HbA1C testing from 25 adult healthy people who came to donate blood in the Blood Transfusion Department of DMCH and 25 critically ill non-infectious cases who were admitted to different wards.

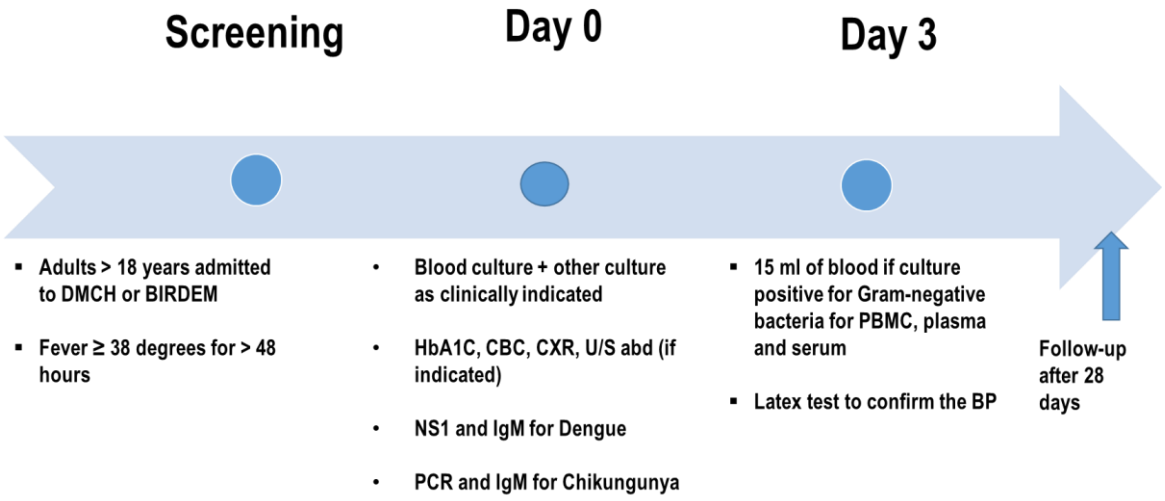

Figure 2.1: Patient enrollment flow chart

The RA followed up the patients until their discharge, and recorded the clinical information. RA assessed the 28-day clinical outcome of the patients via telephone. The attending physician was informed about the patients who were newly diagnosed as having DM based on an HbA1C of 6.5% or above. Such subjects received standard management of DM according to local guidelines. Healthy blood donor volunteers who were found to be diabetic after enrollment (n=1 out of 25), were informed through a letter (Annex 7) in Bangla to see the outpatient department of DMCH for initiating management of DM.

2.1.11. Sample Handling

Up to 30 ml of blood was collected from patients (15 ml in each of two visits if blood culture positive for Gram-negative bacteria). Isolation of PBMC and serum was performed following the protocol described in chapter 2.2. Collected serum, plasma, PBMCs and isolates were stored at -70 °C to -85 °C for further immunological studies in Oxford. World Courier Ltd. is the only carrier to have a licence to ship biological samples from Bangladesh to abroad. Following obtaining all permissions, PBMC,

serum and plasma were shipped to Oxford in December 2017 for further analysis. Samples were stored in Oxford in accordance with the Human Tissue Act (HTA) and local biosafety regulations. Immunological studies included evaluation of cellular immunity to bacteria by flow cytometry, and measurement of antibody response to bacteria by indirect haemagglutination assay. Samples were stored for further tests such as serology, PCR, ELISA, and gene expression to identify the aetiology of fever in adults in Bangladesh.

2.1.12. Data management

Clinical and laboratory data were collected through a structured CRF with no patient-identifying details. All data were stored in an encrypted password-protected database, and the study was conducted in accordance with Good Clinical Practice (GCP). The RA was responsible to collect and record the data. The PI and other co-investigators oversaw the data collection. The PI was responsible for maintaining the required study records, timeliness, completeness and accuracy of the information in the CRF. Data was crosschecked by the PI with original source data every alternate day on a random basis. Approximately 30% of data of the entire study were crosschecked by this way. Hard copies of the data will be kept for 3 years and electronic copies will be kept for a minimum of 5 years after completion of the study.

2.1.13. Quality assurance procedure

The study was conducted in compliance with the protocol and in accordance with GCP. Either the PI or co-investigators directly monitored the entry of each study subject. The PI and the Head of the Microbiology Department of the study hospital (BIRDEM)

monitored the laboratory procedures and specimen collection and storage. The PI also made the study documents and relevant hospital records readily available to the members of the Ethical Committee and fully cooperated during their visit. The REC of BIRDEM visited once during the study.

2.1.14. Statistics and analysis

Data were analysed using the software package GraphPad Prism version 7.0 (San Diego, CA, USA). Two-sided p values were calculated using the Chi-square and Fisher's exact test for dichotomous and ordinal variables. Continuous variables were compared using one-way Anova and the Mann-Witney U-test. Demographic factors and clinical characteristics were summarized with counts (%) for categorical variables and median (interquartile range [IQR]) and mean (±standard deviation) for continuous variables.

2.1.15. Funding

This study was funded by the Commonwealth Scholarship of Dr Fazle Rabbi Chowdhury (BDCS-2015-44) and by the Welcome Trust Fellowship award of Prof Susanna Dunachie (ref WT00174A1A).

2.2. Stimulation of MAIT cells with *B. pseudomallei* and other Gram-negative bacteria

2.2.1. A. Study subjects (*B. pseudomallei* infection)

Prof Dunachie's satellite laboratory at MORU, Sunpasitthiprasong Hospital, Ubon Ratchathani, Thailand recently finished a longitudinal clinical immunology study on human melioidosis (Jenjaroen et al., 2015, Kronsteiner et al., 2019). They enrolled patients and collected blood samples from patients over 18 years of age with culture-confirmed melioidosis at a median of 5 days after admission to hospital (IQR 4-6 days) (Jenjaroen et al., 2015). PBMC were shipped to Oxford on dry ice under a Material Transfer Agreement (MTA). PBMC were used from patients with acute melioidosis (week 0) who survived or died, and from those who survived at follow-up one year later (week 52). 26% of enrolled patients with acute melioidosis (week 0) died within 28 days (28DMort) (Kronsteiner et al., 2019), and 67% had a diagnosis of DM (defined for the purpose of this study as a past medical history of diabetes and/or a blood glycated haemoglobin (HbA1c) of ≥ 7%) (Jenjaroen et al., 2015, Kronsteiner et al., 2019). Short-term (28-days) survival status was determined using hospital mortality records and contact by telephone Jenjaroen et al (Kronsteiner et al., 2019). Healthy endemic control subjects were recruited from the blood donation clinic at Sunpasitthiprasong Hospital. Control subjects were considered as seronegative controls for melioidosis if their IHA titre to BP was <1:40. Healthy unexposed subjects were recruited from the Peter Medawar Building for Pathogen Research, Oxford and their samples were used for titration and blocking experiments.

2.2.1. B. Study subjects (Gram-negative infection)

PBMC from all Gram-negative culture-confirmed cases were collected as part of the aetiology of fever study (see chapter 2.1 for details) from DMC and BIRDEM Hospitals, Bangladesh. Samples were shipped to Oxford on dry ice and analysed. The acute Gram-negative sepsis patients (defined according to SIRS criteria) were admitted between one to three weeks of the course of their illness. PBMC from two control groups were also collected. Healthy endemic control subjects were recruited from the blood transfusion department of DMC. Critically ill non-infectious patients (acute stroke, acute pesticide poisoning, diabetic ketoacidosis, severe acute asthma and acute exacerbation of COPD) were enrolled from the medicine department of DMC.

2.2.2. Ethical permissions

The ethics committees of the Faculty of Tropical Medicine, Mahidol University (TMEC-12-014 and TMEC-15-046), of Sunpasitthiprasong Hospital, Ubon Ratchathani (018/2555 and 017/2559) and the OXTREC (64-11 and 35-15), approved the Thai melioidosis study protocol. The ethics committee (reference mentioned before) of DMC, BIRDEM Hospital and OXTREC, approved aetiology of febrile illness study in Dhaka, Bangladesh. Written informed consent was obtained for all patients enrolled in the study, (including for storage and export to UK). Healthy unexposed subjects recruited in Oxford gave written informed consent, and an NHS Research Ethics Committee (16/YH/0247) approved the work.

2.2.3. Peripheral blood mononuclear cell isolation

PBMCs were isolated from whole blood within three hours of blood draw by density gradient centrifugation (Annex 8) using Lymphoprep (AxisShield, Oslo, Norway), and subsequently cryopreserved with 10% heat-activated fetal calf serum (FCS) and Dimethyl sulfoxide (DMSO). They were transferred to the -80°C freezer using freezing containers, (Nalgene Mr Frosty®) for 24 hours, then subsequently transferred into liquid nitrogen. Cells were shipped to Oxford on dry ice, with courier records confirming confirmation of the cold chain, and then stored in liquid nitrogen until use. Cells were thawed at 37^0C and washed in RPMI 1640 (Sigma, St. Louis, MO, USA) with 10% heat-activated fetal calf serum (FCS; (Life Technologies, Carlsbad, CA, USA), 1% L-glutamine, and 1% penicillin/streptomycin (R10) (all Sigma-Aldrich, Dorset, UK). Cells were washed once in R10 and treated with benzonase (Merck Millipore, Billerica, MA, USA) 1ul/ml of media for 30min at 37ºC, 5% CO2, 95% humidity as per the SOP (Annex 9). After treating with benzonase, cells underwent centrifugation then were re-suspended in R10. Cells were counted using the haemocytometer with trypan blue staining (Sigma) to assess viability for functional assays. For *ex vivo* activation and phenotyping, cells were immediately subjected to flow cytometry staining. For acute BP samples, the derogated category III laboratory was used, with the remainder processed in the category II laboratory. The PBMC of Gram-negative patients' samples were isolated in the microbiology department of BIRDEM hospital following the same protocol (Annex 8), following provision of all the necessary training to the laboratory assistant in Bangladesh by the PI (Dr Chowdhury).

2.2.4. THP-1 cell maintenance and propagation

A monocyte cell line was used as antigen presenting cell (APC) in all the experiments. THP-1 cells (ECACC, UK) were cultured in RPMI 1640 with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin, and 0.05% β- mercaptoethanol (all Sigma-Aldrich, Dorset, UK) at 2x 10^5 cells/ml. After every 20 to 25 passage (based on viability) a new batch of THP-1 cells was thawed and used for experiments.

2.2.5. Co-culture and intracellular staining

THP-1 cells were plated at a density of 80,000 cells per well in a 96-well round-bottom plate, and subsequently stimulated with 2% paraformaldehyde-fixed (PF) *E. coli* at an MOI (multiplicity of infection) of 30 was used for healthy and patient samples, and MOI 15 to 60 for titration experiments (see Figure 4.2 C, D). The DH5-α genotype of *E. coli* (kindly supplied by the Klenerman laboratory) was grown in the category II laboratory (Annex 10) and used for stimulation. For BP stimulation, THP-1 cells were incubated with PF BP at MOI 50 for healthy and patient samples, and MOI 25 to 75 for titration experiments (see Figure 4.2 A, B). Titration experiments were performed to choose the best paraformaldehyde concentration (0.5%) amongst 0.25% to 2% according to MAIT cell number and MAIT-IFN-γ secretion. PF BP K96243 strain (Annex 11) was collected from the microbiology laboratory of MORU, Bangkok, Thailand.

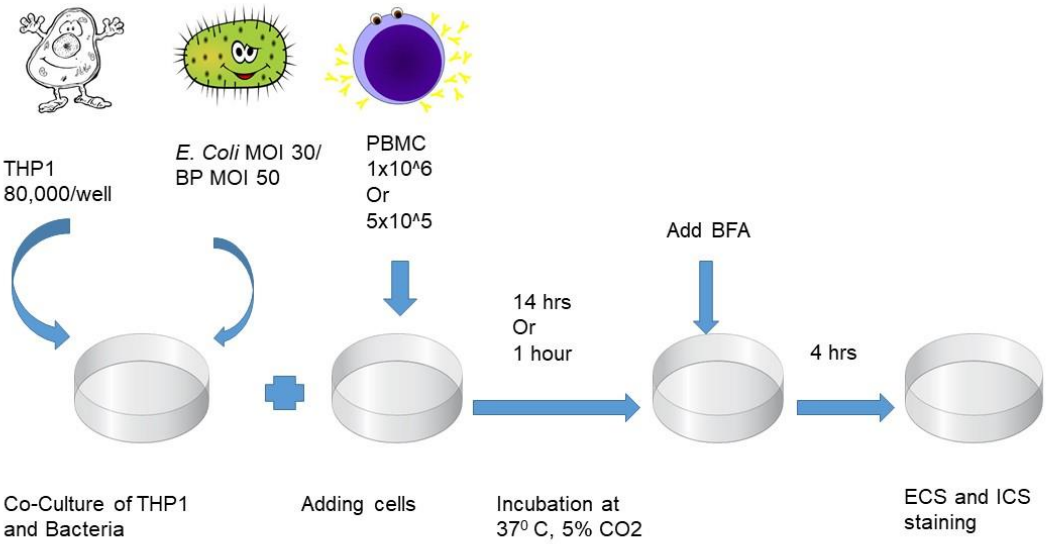

Figure 2.2: Co-culture and staining design of the experiment.

PBMC were added to infected THP-1 cells at 80,000 cells/well and co-cultured overnight (16 hours) (for functional assays; Annex 12) and for one hour (for activation and phenotype assays; Annex 13) at 37°C, 5% CO_2, 95% humidity. Brefeldin (1:1000 final dilution; 20µl/well) was added to each well for the last 4 hours of incubation. Cells were subsequently used for flow cytometry staining (Annex 11 and 12).

2.2.6. Gram-negative bacteria/ antigen

2% PF strains of *E.coli*, *S.* Typhi, *P. aeruginosa*, and *K. pneumoniae* were used for MAIT cell stimulation in the Gram-negative sepsis study. The PF CT18 strain of *S* Typhi was kindly prepared by the Oxford Vaccine Group (OVG) microbiology laboratory led by Prof Andrew Pollard, due to regulatory requirements for *S*. Typhi. The strain was grown, fixed and counted by Dr Jennifer Hill, OVG following our SOP

(Annex 14). ATCC 13368 strain of *K. pneumoniae* and ATCC 27853/ NCTC 12903 strains of *P. aeruginosa* were supplied by the Oxford University Hospitals NHS Foundation Trust microbiology laboratory. The organism was grown, fixed and counted at Medawar by Dr Chowdhury according to SOP (Annex 15 and 16). For *S.* Typhi stimulation, THP-1 cells were incubated at MOI 50 for both controls and patient's samples and MOI 25 to 75 for titration experiment (see Figure 6.1). The MOI (50) and titration experiment was the same for *P. aeruginosa* and *K. pneumoniae* stimulation (see Figure 6.1).

Staphylococcal enterotoxin B (SEB) was used as a positive control in all the experiments. SEB is a standard stimulant used frequently in immunology research laboratories as a positive control. It is a super-antigen and has the ability to bind to class II MHC molecules on antigen presenting cells and stimulate large populations of T cells (Pinchuk et al., 2010).

2.2.7. Fluorescence activated cell sorting (FACS) staining and antibodies/dyes

PBMC were re-suspended in phosphate buffer saline (PBS) and incubated for 20 minutes with near-infrared live/dead fixable stain (Invitrogen, Carlsbad, CA, USA, 1/500) and fluorochrome-conjugated primary human-specific antibodies in the presence or absence (in the functionality experiment) of human FcR blocking reagent (Miltenyi Biotec) at 4ºC. After washing with PBS, cells were resuspended in IC fixation solution (eBioscience, San Diego, CA, USA) or PBS depending on the protocol for intracellular staining. Cells were fixed with 2% fixation/permeabilization solution (BD Biosciences, eBioscience) for 15 min at 4ºC, washed with permeabilization buffer (BD Biosciences, eBioscience) followed by incubation with fluorochrome-conjugated human-specific antibodies in the presence or absence (in functionality experiment) of FcR blocking reagent for 30 minutes. Extra and

intracellular markers were incubated together for functionality experiment and for activation/phenotype experiment extracellular marker followed by the intracellular marker. After washing with permeabilization buffer, the samples were resuspended in 1xPBS and acquired on a MACSQuant Analyser 10 (Miltenyi Biotec) for acquisition. MACSQuant acquisition is based on volume rather than cell count, but the volumes used in these experiments were typically representative of over 200,000 events in 100 ul of PBS (excluding dead cells). The acquisition was performed on the same day for all experiment. Data analysis was performed with FlowJo Version 10 (FlowJo LLC, Ashland, OR, USA) and specific gating strategies can be found in Figure 4.1, 3 and 6. Details of human antibodies/dyes for FACS and their working concentrations are described in table 2.2.1.

Table 2.2.1. Details of Markers and Fluorochromes used in the experiment and their working concentration

Marker	Fluoro-chrome	Company	Working concentration	Comment
Functional				
CD3	PE-Cy5	Bio Legend, UCHT1	1/200	Pan T cells
CD3	eFluor450	eBioscience, 30 OKT3	1/100	
CD4	V 450	BD Horizon, L200	1/160	T helper cell
CD4	VioGreen	Miltenyi, VIT4	1/25	
CD8a	BV 510	Bio Legend, RPA-T8	1/600	Cytotoxic T cells
CD8	PE-Vio770	Miltenyi, BW135/80	1/50	
CD161	PE Cy7	Bio Legend, HP-3G10	1/50	NKT, the majority of NK cells, MAIT, some CD3+
CD161	PE	Miltenyi, 191B8	1/50	
TCR Vα 7.2	APC	Bio Legend, 3C10	1/50	Characteristics of MAIT cells
IFN-Ɣ	PE-Cy7	Bio Legend, 4S.B3	1/40	CTL signature cytokine
IFN-Ɣ	FITC	Miltenyi, 45-15	1/50	CTL signature cytokine
IFN-Ɣ	PE	Bio Legend, 45.B3	1/40	CTL signature cytokine
TNF-α	FITC	eBioscience, Mab11	1/40	Inflammatory cytokine
TNF-α	PerCP/Cy5.5	Bio Legend, MAb11	1/50	Inflammatory cytokine
Activation/ phenotype				
CD69	PE-Vio770	Miltenyi	1/20	Activation marker

Gzm B	FITC	Bio legend, GB11	1/50	Cytotoxic effector molecule
Exhaustion				
PD-1	BV421	Bio legend, EH12.2H7	1/50	Exhaustion marker
TIM-3	APC	Bio legend, F38-2E2	1/50	Exhaustion marker
Blocking experiment				
Anti-MR1		Bio Legend, 26.5	1/100	TCR blocking
Anti-IL-12		Bio Legend, C8.6	1/200	Cytokine blocking
Anti-IL-18		Bio Legend, KU18-81	1/100	Cytokine blocking
Isotype control		Bio Legend, MOPC-173	1/100	Negative control to identify background

2.2.8. Statistical analysis

Statistical analysis and graphical representation were performed using GraphPad Prism Version 7.0 (San Diego, CA, USA). Cytokine data are presented as box and whiskers plots and floating bars with a line at the median to compare between acute survived versus died, acute versus endemic healthy and critically ill non-infectious control, and diabetes versus non-diabetic. Activation and phenotype data are presented as violin plots as median with 95% confidence intervals. Statistical analysis was performed by Mann-Whitney U-test, one-way ANOVA and one-way ANOVA with Tukey's multiple comparison tests for non-parametric data and student's paired two-tailed t-test for parametric data (titration experiments). In all analyses, the statistical significance of the result is indicated where p-value (****$p \leq 0.0001$; ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$).

3. Is melioidosis an important cause of febrile admissions in Bangladesh?

3.1. Introduction

Bacterial and viral infectious diseases are still the leading cause of death in Southeast Asia (GBD Collaborators, 2004). The aetiology of febrile illness remains poorly characterized in many places in LMIC. The causative pathogens are not usually identified, so patients are treated empirically with antibiotics, which carries the twin risks of incorrect treatment and the inappropriate use of antibiotics, which in turn can drive global AMR. Although the global burden of some infections is believed to be substantial (e.g. enteric fever) (Stanaway, 2019), the prevalence of many others, including melioidosis, is less known and frequently under-diagnosed. Bangladesh is an example of a highly populous country where agriculture comprises about 30% of GDP and provides over 90% of rural employment (Mohajan, 2013). Melioidosis is endemic in South east Asia and parts of Australia and may be a significant undiagnosed cause of infection and death in Bangladesh. Since 1961, 51 culture confirmed cases have been reported from Bangladesh (Chowdhury et al., 2018).The clinical presentation of melioidosis is widely varied, and definitive diagnosis requires a skilled microbiology laboratory making it difficult to diagnose in low resource settings. Moreover, gross under-reporting due to lack of awareness occurs. The varied clinical presentation and lack of diagnostic facilities and manpower make this organism a major threat for Bangladesh. Prior to commencement of the study, it was the perception amongst senior professors of infectious disease in Dhaka that melioidosis is seen rarely or not at all in Dhaka (personal communication). In this chapter we looked for the proportion of melioidosis cases among febrile patients admitted to two major tertiary care hospitals in Dhaka, Bangladesh. In addition, we sought to identify the other aetiologies of febrile illness among adults. Recognition of

the specific infectious agent is key for improvements in treatment strategies and antibiotic stewardship.

3.2. Aims

A. To identify the proportion of melioidosis cases among 201 febrile adult patients admitted to DMC and BIRDEM.

B. To identify the proportion of probable sepsis (based on previous sepsis definition due to lack of available parameters such as lactate for current sepsis definition, (Levy et al., 2003) cases among 201 febrile adult patients admitted to DMCH and BIRDEM.

C. To identify the aetiology and define the 28 day clinical outcome of febrile illness in adults admitted to DMCH and BIRDEM.

D. To detect the pattern of antimicrobial resistance in blood culture positive bacteraemia cases.

3.3. Results

3.3.1. Patient enrollment profile and results of different sites of microbiological culture

237 cases were assessed for eligibility, out of which 36 cases were not enrolled. Out of 36 cases, 17 patients did not meet the inclusion criteria and 19 declined to take part. 201 patients were enrolled in this study. Among them 13.9% (28) were positive for microbiological cultures and 86.1% (173) were culture-negative. Out of 28 culture positive cases, 18 (8.9%) were positive for blood culture and 10 (4.9%) were positive for other cultures: urine (5; 2.4%), pus (3; 1.4%) and sputum (2; 0.9%). 4/18 (all BP) patients with a positive blood culture were also culture-positive from an additional clinical site.

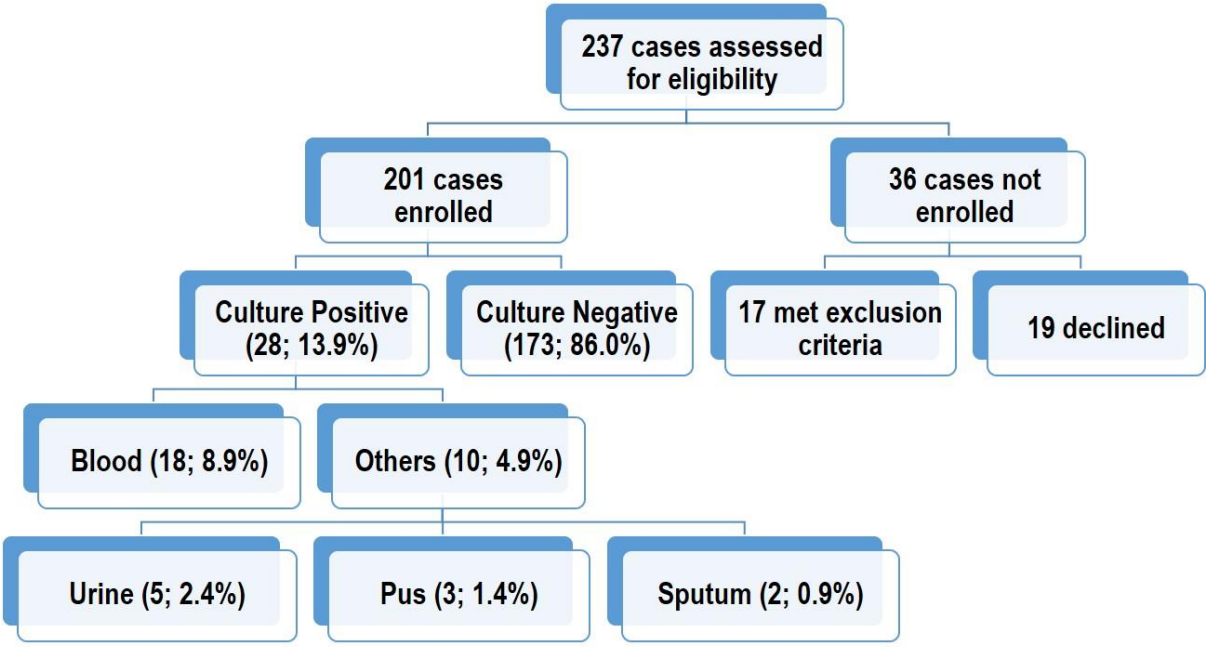

Figure 3.1: Patients' enrolment profile and results of different sites of microbiological culture in adult febrile patients admitted in DMCH and BIRDEM hospital from July to September 2017.

3.3.2. Baseline socio-demographic characteristics of the patients admitted with fever in DMCH and BIRDEM hospital

The patients were divided into two categories (table 3.1): patients with SIRS and patients without SIRS. The presence of two more of the following criteria is considered as SIRS (Levy et al., 2003). The criteria are

- Temperature > 101^0F (38.3°C) or < 96.8^0F (36°C)
- Tachycardia >90 bpm
- Tachypnoea >20 breaths/minute and
- Leucocytosis (WBC count >12×10^9/L) or leukopenia (WBC count <4×10^9/L) (Levy et al., 2003).

The median (IQR) age of the patients was 35 (23 to 55). The patients with SIRS were from a slightly higher age range (38; 24 to 55) compared to the without SIRS group (30; 20 to 44). A greater proportion of patients enrolled were male (56.7%), with a male: female ratio of 1.3:1. The median height and weight of the patients was 167.6 cm and 65 kg. In terms of occupation, 33.3% (67) were a homemaker, followed by student (25.8%; 52), day labourer (20.3%; 41), farmer (13.4%; 27) and others. The approximate distance to hospital from home was higher (155.2 km) in the no SIRS group compared to patients with SIRS (119.9 km) with a mean of 124.7 km considering all patients. The lag period between development of symptoms and hospitalization was 11.34 (± 6.8) days. Amongst all patients, 32.3% (65) had a history of soil exposure. There was no statistical difference between the two groups in any of above parameters.

Table 3.1: Baseline socio-demographic characteristics of the patients admitted to DMCH and BIRDEM with acute febrile illness

Characteristics	All (n=201)	Patients with SIRS± (n= 174)	Patients without SIRS± (n=27)	P value μ
Age (years; median, IQR)	35 (23 to 55)	38 (24 to 55)	30 (20 to 44)	0.524
Male (number, %)	114 (56.7%)	94 (54.0%)	19 (70.3%)	0.144
Height (cm; median, IQR)	167.6 (162.6 to 170.2)	167.6 (162.6 to 170.2)	167.6 (160.0 to 170.2)	0.803
Weight (kg; median, IQR)	65 (60 to 70)	65 (60 to 70)	65 (60 to 70)	0.845
Occupation‡‡				
Homemaker	67 (33.3%)	62 (35.6%)	5 (18.5%)	0.122
Student	52 (25.8%)	42 (24.1%)	10 (37.0%)	0.162
Worker/ day labourer	41 (20.3%)	36 (20.6%)	5 (18.5%)	1.000
Farmer	27 (13.4%)	22 (12.6%)	5 (18.5%)	0.374
Others Ψ	13 (6.4%)	11 (6.3)	2 (7.4%)	0.688
Approximate distance to hospital from home (in km; mean, SD)	124.7 (120.8)	119.9 (121.5)	155.2 (113.2)	0.369
History of soil exposure (number, %)	65 (32.3%)	56 (32.1%)	9 (33.3%)	0.993
Lag period between symptom and hospitalization (in days, mean, SD)	11.34 (6.8)	11.25 (6.7)	11.89 (7.6)	0.905

μ One-way Anova and fishers exact test (two-tailed) was applied
‡‡ includes one missing data
Ψ includes businessman, carpenter, plumber and unemployed.
± SIRS includes two or more of the criteria which includes > 101^0F (38.3°C) or < 96.8^0F (36°C); heart rate >90 bpm; respiratory rate >20 breaths/minute and WBC count >12×10^9/L or <4×10^9/L.

3.3.3. Clinical features, comorbidities and clinical outcome of acute febrile patients

Table 3.2 describes the clinical parameters of the patients, including symptoms, signs, comorbidities and outcomes. In addition to fever, many patients (118; 58.7%) presented with GI symptoms in the form of abdominal pain, diarrhoea and vomiting. The next most common symptom was headache (111; 55.2%), confusion (79; 39.3%), urinary symptoms (70; 34.8%), cough (55; 27.3%), unconsciousness (36; 17.9%), joint pain (23; 11.4%) and others. There were no significant differences between the groups in presenting symptoms (patients with or without SIRS).

The mean (SD) temperature was 39.5 ^{0}C (1.7) with a significant difference (p=0.0001) between the SIRS and non-SIRS groups. Patients with SIRS had higher mean (39.6^{0}C; 2.7) temperature compared to patients without SIRS (39.2^{0}C; 3.1). The mean (SD) systolic and diastolic blood pressure of the patients were 101.3 (17.27) and 63.58 (14.63) mm of Hg respectively. The mean (SD) systolic and diastolic pressure was higher (107.0; 11.37 and 66.30; 10.06) in patients without SIRS compared to patients with SIRS (100.4; 17.87 and 63.16; 15.20 respectively) with no significant difference (p=0.1777 and 0.3574 respectively). The mean (SD) heart rate for all patients was 91.30 (17.24) bpm with a significant (p=0.0478) difference between groups. It was much higher (92.48; 17.80) in patients with SIRS compared to without SIRS (83.70; 10.49). There was no significant (p= 0.1555) difference between the groups in case of respiratory rate. The mean (SD) rate for all patients was 18.05 (3.57). These significant differences are expected, as they are the diagnostic criteria for SIRS and was used to divide the data between the groups.

35.3% patients were smokers in the cohort. The most common co-morbidity was DM (42; 20.8%), followed by hypertension (26; 12.9%), chronic kidney disease (CKD; 19; 9.4%) and others. There was no significant difference between the SIRS and non-SIRS groups for co-morbidities. A total of 25.3% (51) patients died and 74.6% (150) survived without any difference between patients with or without SIRS.

Table 3.2: Clinical features, comorbidities and short term clinical outcome of the patients admitted to DMCH and BIRDEM with acute febrile illness

Characteristics	All (n=201)	Patients with SIRS (n= 174)	Patients without SIRS (n=27)	P value μ
Clinical symptoms				
GI symptoms€ (number; %)	118 (58.7%)	103 (59.1%)	15 (55.5%)	0.834
Headache (number; %)	111 (55.2%)	93 (53.4%)	18 (66.6%)	0.218
Confusion (number; %)	79 (39.3%)	66 (37.9%)	13 (48.1%)	0.133
Urinary symptom (number; %)	70 (34.8%)	65 (37.3%)	5 (18.5%)	0.080
Cough (number; %)	55 (27.3%)	44 (25.2%)	11 (40.7%)	0.106
Unconsciousness (number; %)	36 (17.9%)	31 (17.8%)	5 (18.5%)	1.000
Joint pain (number; %)	23 (11.4%)	17 (9.7%)	6 (22.2%)	0.095
Neck Stiffness (number; %)	11 (5.4%)	10 (5.7%)	1 (3.7%)	1.000
Skin/soft tissue abscess (number, %)	4 (1.9%)	4 (2.2%)	0 (0%)	1.000
Rash (number, %)	2 (0.9%)	1 (0.5%)	1 (3.7%)	0.251
Clinical signs				
Temperature (°F; mean, SD)	39.5 (1.7)	39.6 (2.7)	39.2 (3.1)	0.261
Heart rate (bpm; mean, SD)	91.30 (17.24)	92.48 (17.80)	83.70 (10.49)	0.047
Systolic BP (mmHg; mean, SD)	101.3 (17.27)	100.4 (17.87)	107.0 (11.37)	0.177
Diastolic BP (mmHg; mean, SD)	63.58 (14.63)	63.16 (15.20)	66.30 (10.06)	0.357
Respiratory rate (RR; mean, SD)	18.05 (3.57)	18.24 (3.73)	16.81 (2.02)	0.155
Comorbidities				
DM (number, %)	42 (20.8%)	39 (22.4%)	3 (11.1%)	0.046
Hypertension (number; %)	26 (12.9%)	24 (13.7%)	2 (7.4%)	0.540
CKD (number; %)	19 (9.4%)	18 (10.3%)	1 (3.7%)	0.479
Previous Stroke (number; %)	7 (3.4%)	7 (4.0%)	0 (0%)	0.596
Chronic Lung Dis.± (number; %)	5 (2.4%)	4 (2.2%)	1 (3.7%)	0.517
CLD (number; %)	1 (0.4)	0 (0%)	1 (3.7%)	0.134
Malignancy (number; %)	1 (0.4%)	1 (0.5%)	0 (0%)	1.000
Clinical Outcome Ψ				
Survived (number, %)	150 (74.6%)	130 (74.7%)	20 (74.0%)	1.000
Died (number, %)	51 (25.3%)	44 (25.2%)	7 (25.9%)	1.000

μ One-way Anova and fishers exact test (two tailed) was applied
CKD= Chronic kidney disease; **CLD**= Chronic liver disease
± includes chronic obstructive pulmonary diseases and chronic asthma
Ψ clinical outcome included up to 28 days follow up
€ GI symptoms includes abdominal pain, diarrhoea and vomiting

3.3.4. Baseline laboratory parameters of the patients admitted with acute fever

The violin plot (Figure 3.2) demonstrates the comparison of baseline laboratory parameters between patients with and without SIRS. Total white blood cell (WBC) count (Figure 3.2 A) in cells/micro litre (median) was significantly higher (p=0.0001) in patients with SIRS compared to without SIRS (16,550 vs 8,630). Absolute count (in %) of neutrophils (median; Figure 3.2 B) was significantly higher (p=0.0001) in SIRS patients (84%) compared to other patients (70%). The absolute count (in %) of lymphocytes (median; Figure 3.2 C) was significantly lower (p=0.0001) in patients with SIRS (10%) compared to non-SIRS (22%). This significant difference in WBC count is expected, as neutrophilia and lymphopenia are well described in sepsis (Levy et al., 2003).

There was no significant difference (Figure 3.2 D-F) between the groups (SIRS vs non-SIRS) in the haematocrit level (in %), total platelet count (cells/ micro litre) or haemoglobin level (in gm/dl). The median value was 35.10 vs 38.40, 260,000 vs 250,000 and 12.50 (in both cases) respectively. The HbA1c level was significantly higher (p=0.0384) (6%) in patients with SIRS (Figure 3.2 G) compared to other patients (5.8%) but there was no difference (Figure 3.2 I) in serum creatinine level (1.0 mg/dl in both the cases). The ESR level was higher (p=0.0030; Figure 3.2 H) in patients with SIRS (55 mm in 1st hour) compared to without SIRS (35 mm in 1st hour).

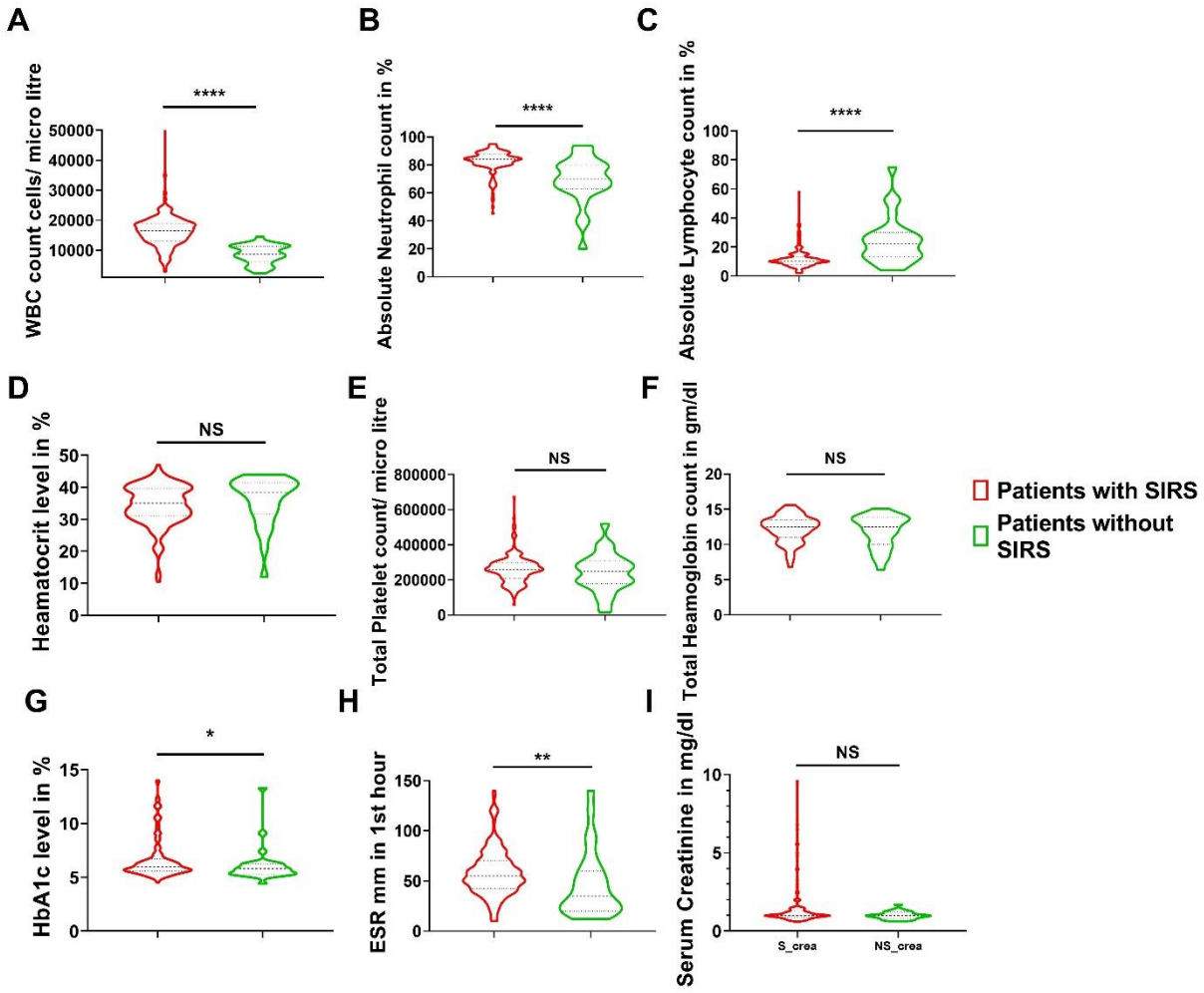

Figure 3.2: Different laboratory parameters and comparison between adult febrile patients with or without SIRS. A. WBC count. **B.** Absolute neutrophil count. **C.** Absolute lymphocyte count. **D.** Haematocrit level. **E.** Total platelet count. **F.** Total haemoglobin count. **G.** HbA1c level. **H.** ESR level. **I.** Serum creatinine level. Data is presented as violin plot (with a line at maximum, minimum and median). Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

3.3.5. Clinical diagnosis and laboratory confirmation of the cases

Figure 3.3 A displays the clinical diagnosis of the cases based on available clinical and laboratory information at the time of discharge, as determined by the PI. The commonest diagnoses were enteric fever (81; 40.2%) and genito-urinary infection (51; 25.3%). The diagnosis of “enteric fever” was a clinical diagnosis assigned on the basis of available clinical information & laboratory reports, but in the absence of culture confirmation. Other diagnoses were sepsis not specified (25; 12.4%), pyrexia of unknown origin (PUO, 15; 7.4%), meningoencephalitis (11; 5.4%), melioidosis (6; 2.9%), lung abscess (7; 3.4%), liver abscess (4; 1.9%) and others (12; 5.9%). ‘Sepsis not specified’ were the patients who presented with the features of SIRS but no source of infection was identified. PUO described as fever without features of SIRS and no source of infection identified. Other causes included TB, dengue fever, septic arthritis, aspiration pneumonia, acute gastro-enteritis, spontaneous bacterial peritonitis and Guillain-Barré syndrome.

15.9% (32) cases were confirmed through microbiological cultures and other tests (Figure 3.3 B). Dengue was confirmed by positive NS1 test and TB confirmed by positive histopathology and serology. In 84.0% (169) cases, the specific aetiological agent could not be identified. Out of the 32 cases where the aetiological agent was confirmed (Figure 3.3 C), each of six were BP and *E. coli*. *S.* Typhi was found in four and *P. aeruginosa* in three cases. Two cases were identified as *Acinetobacter* sp, *K. pneumoniae*, *Staphylococcus aureus*, TB and Dengue. Mixed bacterial growth was reported in three cases, where one was identified as micrococcus of uncertain significance, and the other two were considered contamination.

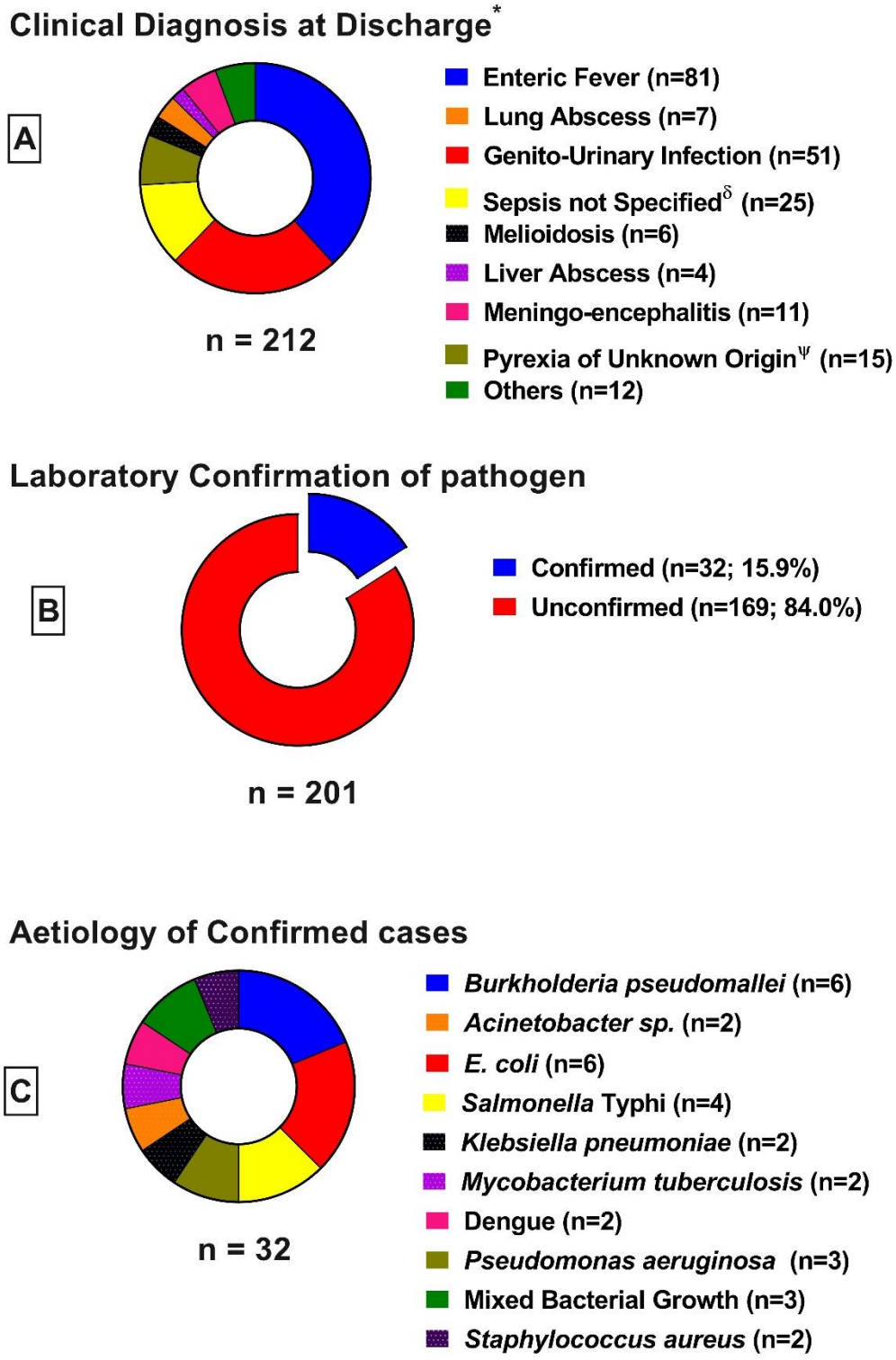

Figure 3.3: Clinical diagnosis and laboratory confirmation of the cases. A. Diagnosis made during discharge based on available clinical information and laboratory results. * Some patients have dual diagnosis. δ Sepsis not specified: SIRS + no source identified. Ψ PUO: fever without features of SIRS+ no source identified. **B.** Confirmation includes all cases positive for blood, urine, sputum and pus culture. **C.** Aetiology of the confirmed cases (n=32).

3.3.6. Demographic and clinical characteristics of melioidosis cases

Table 3.3 describes the demographic, socioeconomic and clinical characteristics of the six melioidosis cases admitted during the study period at DMCH and BIRDEM Hospital. The age of the cases ranged from 32 to 70 years. Three were male and three were female. The distance to hospital from home ranged from 60 km to 180 km. The lag period between symptom and hospitalization ranged between 8 days to 30 days. The majority (4 out of 6) presented as sepsis without any specific focus. All the patients had DM. In 66.6% (4/6) cases, the organism was confirmed in blood. All patients received ceftriaxone before admitted to the hospital. Four patients received Meropenem and two Ceftazidime. Five out of six (80%) of the melioidosis cases died in this study.

Table 3.3: Baseline demographic and clinical characteristics and outcome of melioidosis cases admitted to DMCH and BIRDEM

Variables	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Age (in years)	50	32	42	38	57	70
Sex	Female	Male	Male	Female	Male	Female
Height (in cm)	158.4	170.1	176.	165.1	175.2	170.1
Weight (in kg)	45	70	75	55	70	65
District	Gazipur	B.Baria	Tangail	Cumilla	Gazipur	Feni
Occupation	Housewife	Day labour	Day labour	Housewife	Farmer	Housewife
Approximate distance to hospital from home (in km)	60	150	100	150	150	180
Lag period between symptom and hospitalization (in days)	15	30	14	30	8	30
Exposure to soil	Yes	No	No	Yes	Yes	No
Primary clinical manifestations	Sepsis without focus	Sepsis without focus	Sepsis without focus	Joint pain	Sepsis without focus	UTI
Co-morbidities[ǂ]	DM, CKD	DM	DM	DM	DM	DM
Source of diagnosis	Blood	Blood	Blood	Pus	Blood	Urine
Prior receive of antibiotic	Ceftriaxone	Ceftriaxone	Ceftriaxone	Ceftriaxone	Ceftriaxone	Ceftriaxone
Antibiotics received after admission	Meropenem	Ceftazidime	Meropenem	Ceftazidime	Meropenem	Meropenem
Outcome [Ψ]	Died	Died	Died	Died	Died	Survived

ǂ Co-morbidities: CKD= Chronic kidney disease; DM= Diabetes mellitus
Ψ Clinical outcome included up to 28 days follow up

3.4. Discussion

In this study, we found BP to be the joint commonest cause (2.9%) of bacteraemia in Dhaka, Bangladesh along with *E.coli*. This is despite the fact that Dhaka is a large modern city with concrete covering the ground rather than having soil. A recent modelling study estimated around 16,931 cases annually in Bangladesh with a case fatality rate of 56% (Limmathurotsakul, 2016). My study provides support that melioidosis is a significant cause of disease and death in Bangladesh. It is likely that a higher number of cases would be found if enrolment of bacteraemic patients from melioidosis hotspots elsewhere in Bangladesh such as Gazipur (Annex 17) could be performed. Our study was conducted in 2017, and in 2018, outside of a study setting, the same laboratory at BIRDEM identified six cases of BP (personal communication), which further validates our findings and shows evidence of capacity building by our study. An earlier environmental study in Bangladesh confirmed the presence of the bacteria in soil (Jilani et al., 2016), and another study demonstrated up to 45% sero-positivity by IHA in soil-exposed populations in Bangladesh (Maude et al., 2012). A systematic review on community acquired bloodstream infections in south and Southeast Asia identified BP positive culture in 0.7% cases (Deen et al., 2012). This review included two studies out of a total of 308 cases with a positive blood culture from Bangladesh (Arifeen et al., 2009, Brooks et al., 2007).

Overall, microbiological culture from any site was positive in 13.9% of patients and blood culture was positive was 8.9% in this study, which is lower (15.7% in South East Asia and 15.9% in south central Asia) compared to the regional data in a recent systematic review (Prasad et al., 2015). Considering individual countries, the frequency of positive blood culture status among acute febrile patients in South and

Southeast Asia is quite varied (Prasad et al., 2015, Shrestha et al., 2018). Two studies in Nepal identified 15.6% (Murdoch et al., 2004) and 28.1% (Blacksell et al., 2007) positive culture rate in blood among adult patients. In contrary, the frequency was 8.1% (Singh et al., 2014) and 8.0% (Chrispal et al., 2010) North India and South India respectively which is similar to our findings. In Southeast Asia the frequency of positive culture was 15% in Indonesia ((Punjabi et al., 2012) and 6.3% in Cambodia (Kasper et al., 2012). A study performed in Bangladesh in 2012 identified 19% blood culture positive isolates, which is almost double of our findings. However, comparing blood culture positivity rates in different settings does not allow for differences in frequency and timing of blood culture taking, nor adjustment for whether antibiotics were received prior to blood draw. It is common practice in Bangladesh that people start antibiotics if they have a high fever for more than 2 days. Moreover, people come late to the hospital or attend a community practitioner. People can get a wide range of antibiotics (even intravenous) over the counter without prescription. Around 95% (191) of the enrolled patients received a third generation cephalosporin (ceftriaxone) before admission in this study (to which BP is partially susceptible). We therefore underestimate the burden of bacteria and skewed the frequency in this study. We isolated few species that were sensitive to ceftriaxone, and no *Streptococcus pneumoniae*. Further explanations for the variation in blood culture positivity rates include differences in pattern of patients presenting to hospital, and variations in the rate of non-bacterial causes of fever.

The majority of the fever patients were midlife (median 35 years) and belonged to the most active age group in society. The findings are similar to another study in India (37.4 years) although a lower median age was seen in Nepal (20 years) and in another previous study in Bangladesh (19.8 years) (Abhilash et al., 2016, Blacksell et al., 2007, Faruque et al., 2012). These median ages are very low compared to

hospital admissions in HIC, where most people with bacteraemia are elderly (over 65 years of age). Male predominance was seen in our study, which was also common in other national and regional studies (Abhilash et al., 2016, Maude et al., 2016, Murdoch et al., 2004). Possible reasons for the male predominance include the fact that males are more involved with outdoor activities in Bangladesh, and access to healthcare for females can be restricted for different cultural and socioeconomic factors (Hossen and Westhues, 2011). The majority of females admitted to the hospital were homemakers and this was the same in India (Abhilash et al., 2016). The average distance to hospital from home and mean duration of fever was high in this cohort because most of the patients came from outside of Dhaka, referred because of lack of improvement and lack of diagnosis, and used time for preparation to come to the capital.

The commonest clinical features in patients admitted with fever in our study, such as urinary symptoms, headache, GI symptoms, joint pain and others are similar to other studies reported from Bangladesh and other South Asian countries (Abhilash et al., 2016, Faruque et al., 2012, Murdoch et al., 2004). A considerable number of patients in this study presented with confusion (39.3%) and unconsciousness (17.9%). This is probably because 86.5% (174) patients in this cohort presented with two or more features of SIRS (Levy et al., 2003). In addition, for the same reason mean temperature (39.5^0C) and heart rate (91.30 bpm) was high compared to other fever aetiology studies performed in Bangladesh. Mean temperature was 37.9^0C (Faruque et al., 2012) and 37.6^0C (Faruq et al., 2013) in the previous studies. The systolic (106 and 115 mm of Hg) and diastolic (68 and 74 mm of Hg), blood pressure was higher in those previous studies compared to this study (101.3 and 63.58 mm of Hg), which support our cohort of patients being sicker than the cohorts of the previous two studies. DM is a major risk factor for melioidosis, TB and Gram-negative infections

caused by *E coli* and *Klebsiella* (Dunachie and Chamnan, 2018). Almost a quarter (20.8%) of the adult patients in this study presenting with fever had DM. DM is an emerging and rapidly growing concern because there is now an estimated 8.4 million people with DM currently living in Bangladesh (International Diabetes Federation, 2017). A similar study performed in India also found DM to be an important risk factor in patients with scrub typhus (14.6%), enteric fever (10.6%), leptospirosis (25%) and undiagnosed fever (12.8%) (Abhilash et al., 2016). We also identified a considerable number of patients currently smoking (71; 35.3%) and as hypertensive (26; 12.9%), although it was not recorded in other national and regional studies.

In this study, the median WBC count was 15,800 cells/ml, neutrophil 84% and lymphocyte 11%. The WBC and neutrophil level were higher in our study compared to previous studies performed in Bangladesh (mean 12,656; 68%), India (median 6,700), Laos (median 13,000; 75%) and Vietnam (median 6,500; 67%) (Abhilash et al., 2016, Faruque et al., 2017, Hoa et al., 1998, Phetsouvanh et al., 2006). However, the median platelet count was lower in adult febrile patients from India (157,000) and Vietnam (160,000) compared to ours (260,000) (Abhilash et al., 2016, Hoa et al., 1998), but for the Indian study 30.6%, patients were diagnosed as dengue, making direct comparison less appropriate (Abhilash et al., 2016). The level of haemoglobin, haematocrit and ESR was similar between the Bangladeshi and other south Asian studies (Abhilash et al., 2016, Faruque et al., 2012, Faruque et al., 2017, Hoa et al., 1998).

Previous studies in Bangladesh (Maude et al., 2016) and Nepal (Murdoch et al., 2004) identified enteric fever (17.3% and 27% respectively) as the commonest clinical diagnosis. Shrestha *et al.* also reported typhoid fever as one of the most frequently reported clinical causes of fever (20%) in South and Southeast Asia after reviewing 100 studies (Shrestha et al., 2018). This review also identified dengue,

leptospirosis and typhus as the other commonest causes (Shrestha et al., 2018). Another systematic review on aetiology of severe febrile illness in LMIC also identified enteric fever and malaria as the top most cause of infection (Prasad et al., 2015). However, identification of BP was not optimised in these earlier studies. We did not test for malaria, typhus and other of the above infections due to limited resources. This is one of the limitations of this study. However, malaria is not a major cause of fever in Dhaka and surrounding regions (Haque et al., 2009). Other common clinical syndromes in Bangladesh are urinary tract infection, sepsis without focus and meningoencephalitis and the picture was similar compared to other studies (Maude et al., 2016, Murdoch et al., 2004).

Among the culture-positive cases, *S.* Typhi was the third most common isolate after BP and *E. coli*, followed by *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter spp* and *Staphylococcus aureus*. This is in keeping with other studies in Bangladesh (Maude et al., 2016), Nepal (Murdoch et al., 2004), Laos (Phetsouvanh et al., 2006) Thailand (Lim et al., 2016) and Vietnam (Hoa et al., 1998) except the additional presence of *Streptococcus pneumoniae* in other studies. Reviews on bacterial infection in LMIC and in south and Southeast Asia also confirmed the same organisms as the commonest cause (Deen et al., 2012, Prasad et al., 2015). The reason for the absence of *Streptococcus pneumoniae* in this series is probably that 95% of our patients received ceftriaxone before being admitted to the hospital. There is evidence of AMR within this population, particularly with ESBL producing *E. coli* (3/6; 50%) and *Klebsiella* (2/2; 100%). *E. coli* (4/6; 67%) was also resistant to nalidixic acid/ ciprofloxacin (Annex 18). Nalidixic Acid Resistant *Salmonella* Typhi (NARST; 3/4; 75%) with MDR (resistant to chloramphenicol, ampicillin and cotrimoxazole) typhoid (3/4; 75%) and MRSA (1/2; 50%) was also evident. Susceptibility was defined measuring the zone diameter according to the

CLSI M100, performance standards for antimicrobial susceptibility testing, 28th edition, 2017 (CLSI, 2017). For example, CLSI zone diameter of susceptibility for *E. coli* is ≥26 mm for Ciprofloxacin, ≥23 mm for Ceftriaxone, ≥18 mm for amoxiclav and others (CLSI, 2017). This picture is alarming and is similar to a recently published systematic review from Bangladesh (Ahmed et al., 2019). However, these are very small number of isolates, skewed by being collected in patients referred to a tertiary referral centre after not responding to ceftriaxone and other first-line antibiotics, and large-scale surveillance is required to quantify the prevalence of AMR in this population. Regional studies on antimicrobial susceptibility are also similar (Lim et al., 2016). BP was diagnosed by culture and antibiogram. Any Gram-negative bacilli or non-pseudomonas aeruginosa that are oxidase-positive, typically resistant to aminoglycosides (e.g., gentamicin), colistin, and polymyxin but sensitive to amoxicillin/clavulanic acid was considered as BP. The organism is further confirmed by latex test and treated with Ceftazidime or meropenem on the basis of clinical treatment guidelines (Dance, 2014).Our findings are supported by another study published from the same laboratory (Dutta et al., 2017). Ceftazidime or carbapenem are the mainstay of treatment for the acute phase and amoxicillin/clavulanic acid (co-amoxiclav) could be used as second-line therapy (Dance, 2014). Trimethoprim + sulfamethoxazole is preferred for the eradication phase, with the alternative of co-amoxiclav and doxycycline (Dance, 2014, Maloney et al., 2017). We treated four of our patients with injection of meropenem 1 gram intra-venous 8 hourly and two of our patients with injection of Ceftazidime 500 mg intra-venous 8 hourly. However, five of them died in the first week of starting treatment. The mortality was 25.3% in this study, which was quite high compared to previous studies in Bangladesh (9.7%), Laos (11%) and Vietnam (6%) (Hoa et al., 1998, Maude et al., 2016, Phetsouvanh et al., 2006). This is probably because our patients were tertiary referral, were clinically

more unwell (86% SIRS) than the other studies and insufficient number of ICU beds at the study sites.

3.5. Limitations

Dhaka is concrete, whereas melioidosis is a soil-borne disease. Ideally, we would conduct the study in suspected hotspots such as Gazipur (Chowdhury et al., 2018, Jilani et al., 2016). However, first we needed to establish laboratory capacity. Moving forward from this study, the next step having now established laboratory capacity is to conduct a systematic study in more rural areas of Bangladesh beyond Dhaka. The PI will be based at Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka in the future and will have the opportunity to coordinate such studies if resources are available.

We did not record GCS for the patients, which is an important parameter to assess sepsis. Although the definition of sepsis using the SOFA score has now been internationally accepted (Shankar-Hari et al., 2016), we were unable to use this scoring system because some key parameters such as serum lactate level and mean arterial pressure were unavailable in this resource-limited setting. In our opinion, it is a limitation of the SOFA score that it relies on measurement of parameters not readily available in low-resource settings. We also could not perform testing for malaria and typhus due to lack of resources. Paired serum analysis and PCR would be ideal to include for fever aetiology study in future. Melioidosis is not a notifiable disease in Bangladesh although it is a Bioterrorism Cat III organism (Currie and Kaestli, 2016). Clinical management of melioidosis is not standardized in Bangladesh and no treatment guidelines are available based on local antibiogram, but international

guidelines for the management of melioidosis can be used (Currie Seminars in Infection). Unfortunately, only this single centre in Bangladesh (BIRDEM) can currently diagnose BP infection (Chowdhury et al., 2018), but my on-going work aims to increase capacity for BP diagnosis in Bangladesh.

3.6. Conclusions

This study highlights the existence of melioidosis as a significant cause of fever and mortality in Bangladesh, with BP the joint commonest bacteria, alongside *E. coli*, isolated from adult patients with fever admitted to two tertiary referral hospitals in Dhaka, Bangladesh. This confirms the recent modelling study that estimated the global burden of melioidosis (Limmathurotsakul et al., 2016a). Evidence of AMR within other Gram-negative bacteria in Bangladesh was also found. An awareness-building program for melioidosis targeting clinicians, laboratory training for microbiologists and preventive educational programs for the public is urgently required in Bangladesh.

4. MAIT cells are important in host defence against BP infection

4.1. Introduction

MAIT cells are non-classical innate-like T cells and have both innate and adaptive immune effects (Howson et al., 2018). MAIT cells comprise up to 5% of the total T cell and 0.1% of the iNKT cell population in the human body (Ussher et al., 2014a). These cells are abundant in the blood circulation and comprise 1-10% of peripheral blood T lymphocytes (Kurioka et al., 2015, Ussher et al., 2014b). Microbes that synthesise riboflavin precursors (Gram-negative bacteria, Yeasts) are able to stimulate MAIT cells in an MR1-dependent manner (Reantragoon et al., 2016). In an animal model, it has been demonstrated that MR1/MAIT cell-deficient mice are more prone to infection by *K. pneumoniae* and TB (Chua et al., 2012, Georgel et al., 2011). All these discoveries suggest that MAIT cells could be important in controlling bacterial infection particularly those possessing riboflavin precursors in their synthetic pathway, but there are minimal human data.

In humans, CMI to *Burkholderia* is found to be associated with IFN-γ, IL-12, IL-18 and TNF-α (Koh et al., 2013, Tippayawat et al., 2009). In Northeast Thailand, 200 acute melioidosis cases and their subsequent follow up showed strong CD4+ and CD8+ T cell responses and a direct relationship of anti-bacterial T cell responses with mortality (Jenjaroen et al., 2015). In particular, the very low CD8+ cells seen in fatal cases support the role of CD8+ T-cell responses in defence against BP. The MAIT cell response to BP has never been reported in the literature. Elucidation of the role of MAIT cells in protection against disease and death from melioidosis is important for the development of an effective vaccine and novel therapeutics. Understanding the protective pathways will guide developing the optimal effective vaccine platform and

adjuvant. It would also help to develop robust immunogenicity assays for vaccine trials, and open opportunities for the development of future therapeutic strategies targeting MAIT cells. In this chapter, we hypothesised that the frequency of MAITs and IFN-γ secretion by MAITs will be lower in acute melioidosis patients who died compared to survivors. We also hypothesised that the MAIT cell response will be lower in acutely ill patients compared to healthy endemic subjects.

4.2. Aims

A. To identify the phenotype, activation and function of MAIT cell during acute melioidosis with distinct clinical outcomes (Survived versus Died).

B. To compare the phenotype, activation and function of MAIT cells between patients with acute melioidosis and the healthy population in the endemic region.

C. To track the pathway of IFN- γ secretion after MAIT cell activation.

4.3 Results

4.3.1. MAIT cell responses to BP

A flow cytometry panel to identify MAIT cells was selected and optimised using the published literature (Kurioka et al., 2015, Ussher et al., 2014a) and experience from researchers in Professor Paul Klenerman's group at the Peter Medawar Building for Pathogen Research, Oxford. PBMC from healthy Oxford volunteers were used in initial experiments. MAIT cells were characterised as CD161++ Vα7.2+ in different live T cell populations including CD4+, CD8+ and DN T cells (elaborated in Figure 4.3). PF BP

and *E. coli* as well as SEB were used as stimulants in all the experiments. *E. coli* and SEB are considered as positive controls, and media (R10) as a negative control in all the experiments. Initially, the staining protocol was optimised. In the Klenerman laboratory, a combined staining protocol (Annex 12) is employed in which antibodies towards both extra- and intracellular markers are added simultaneously to previously PF and permeabilised cells. In order to optimise our approach, this work set out to compare this combined staining approach with the two-stage separate staining approach which is the practice in our laboratory, whereby extracellular markers are stained with fluorochrome conjugated antibodies first on live cells followed by fixation, permeabilisation and subsequent staining with antibodies towards intracellular markers (separate staining; Annex 12). The gating strategies for the separate (Figure 4.1A) and combined (Figure 4.1B) staining protocols are shown below.

The sensitivity of a combined FACS staining versus a separate FACS staining protocol to measure MAIT cells and MAIT induced IFN-γ secretion was evaluated (Figure 4.1.C and D). The combined staining was found to be significantly better for identification of CD8+ MAIT cells (in %) and subsequent IFN-γ production (in %) in *E. coli* stimulated PBMC (MAIT p= 0.03, IFN-γ p=0.001). Differences were not significant for BP and SEB. Although this benefit was only evident for *E. coli,* the combined staining protocol was selected for use for all subsequent functional experiments to ensure comparability of results from different stimulants. For all *ex vivo* activation, exhaustion and phenotyping experiments, the separate staining protocol was used, the reason being that the binding of some antibodies to their target including the activation marker CD69 are sensitive to the fixation process.

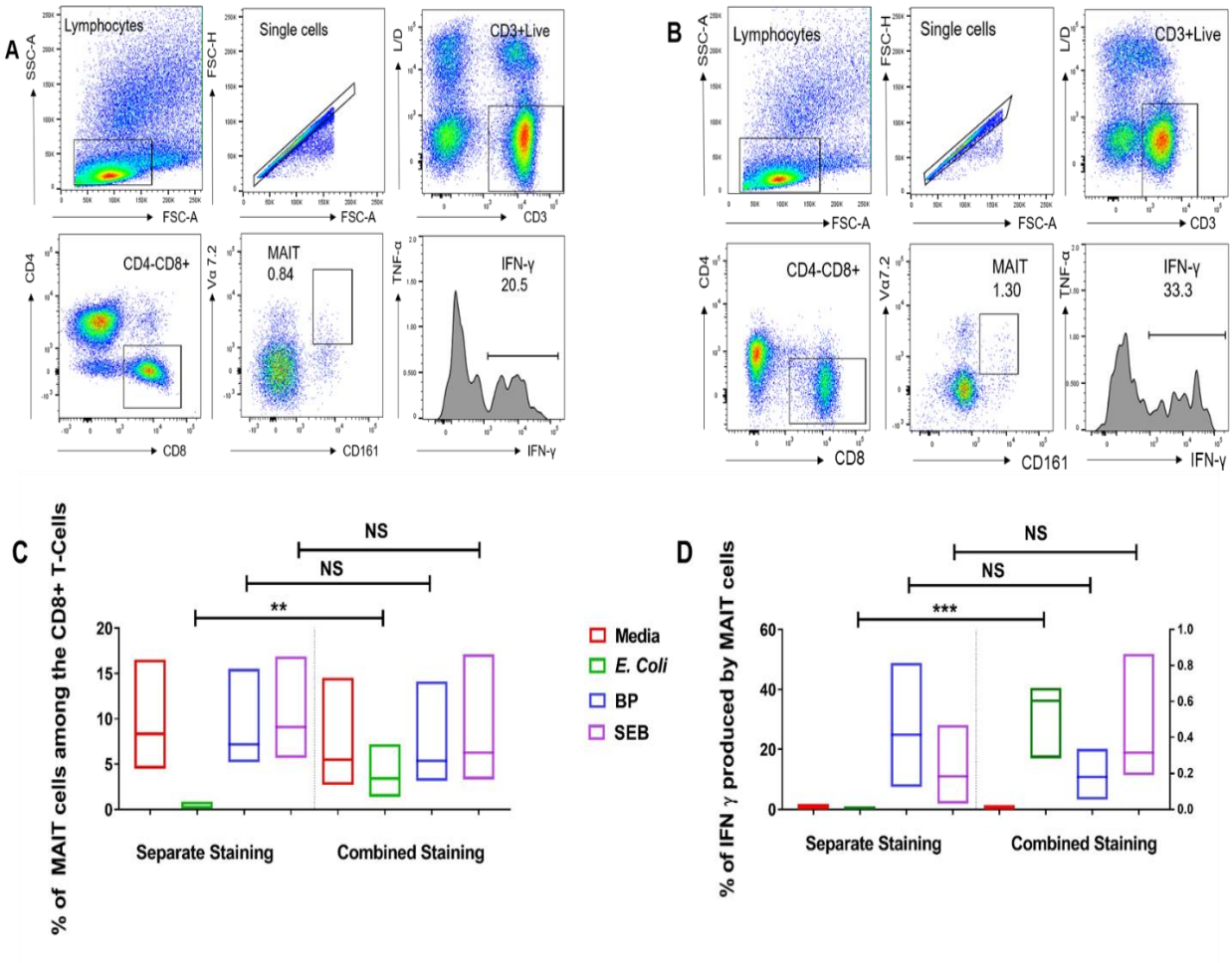

Figure 4.1. T cell gating strategy and comparison between separate and combined FACS staining protocol. A. Separate staining and **B.** Combined staining of a representative *E. coli* stimulated sample. Gating was performed on the Lymphocyte population, followed by single cell gating. Within the CD3+ live cell gate different T cell populations were identified based on CD4 and CD8 expression. Subsequent gating for CD161++ and Vα 7.2+ identifies MAIT cells in the respective T cell populations. MAIT cells were further gated for IFN-γ and TNF-α. **C.** Percentage of MAITs amongst the CD8+ T cells and **D.** Percentage of IFN-γ produced by CD8+ MAITs comparing the two different protocols. Data is presented in floating bars with a line at the median. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

4.3.2. Optimizing the concentration of BP and *E. coli*.

Once the ideal FACS staining protocol had been determined, a titration experiment of the stimulants (BP and *E. coli*) was performed to identify the optimal concentration to stimulate IFN-γ secretion by MAIT cells. PF BP and *E. coli* were used in titration and subsequent experiments. Different MOIs were tested in MAIT IFN-γ assays to determine the optimal dose for BP and *E. coli* , i.e. MOI 25, 50, 75 and MOI 7.5, 15, 30, 60 respectively. In the case of BP, the greatest number of CD8+ MAIT cells and IFN-γ expression thereof were found with MOI 50 (50 BP per THP1 cells) (Figure 4.2 A and B), although the difference did not reach statistical significance compared to other MOIs. Titration experiments for different PF concentrations (2, 0.5, 0.25, and 0.125) used for BP fixation were also performed. Higher CD8+ MAIT cell frequency was observed for 0.5 % PF fixed BP compared to other PF concentrations (Figure 4.2. C). MAIT-induced IFN-γ production was also significantly higher when using the 0.5% PF fixed BP preparation (Figure 4.2.D).

For *E. coli*, the highest frequency of MAIT cells was seen for MOI 15, without any significant difference to other MOIs (Figure 4.2.E). The highest percentage of IFN-γ secretion from CD8+ MAIT cells was seen for MOI 30 (Figure 4.2.F). This is also in accordance with the findings Prof Klenerman's laboratory. Based on these findings, BP MOI 50 and *E. coli* MOI 30 were selected for all subsequent experiments.

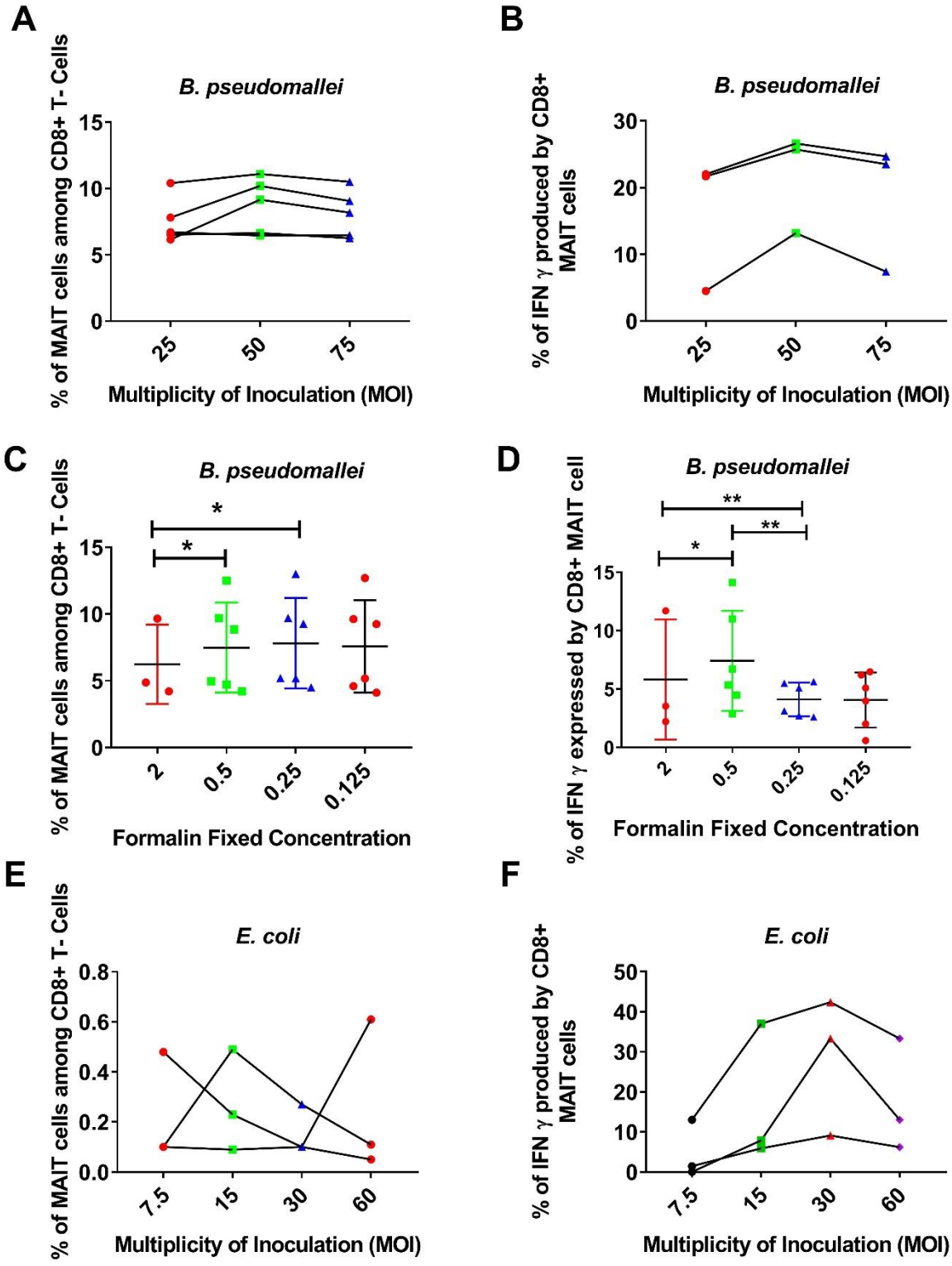

Figure 4.2. Percentage of MAIT cells and MAIT induced IFN-γ produced by CD8+ T cells after stimulation with BP **(A and B)** and *E. coli* **(E and F)** using different MOI. **C &D**, Comparison between different formalin fixed BP. Each Line and dot represent individual donors. Student's paired two-tailed t-test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

4.3.3. Gating strategy to identify MAIT cells in different T cell populations

Gating was performed on the lymphocyte and T cell populations as shown in Figure 4.1. MAIT cells were characterised as CD161++ Vα7.2+ within CD4- CD8+ positive (Figure 4.3 A), CD4- CD8- (DN; Figure 4.3 B), CD4+CD8- (Figure 4.3 C) and the combined CD4-CD8+ and DN (Figure 4.3 D) live T cell populations. Their effector function was assessed by gating on all IFN-γ+ cells (see Figure 4.3 B for gating strategy) within the MAIT cell gate. Since almost all the MAIT cell population was seen in the CD4- CD8+ positive and DN population (Reantragoon et al., 2013), I have analysed them together as a 'combined' compartment in this thesis as an overall representation of MAIT cells, along with separate analysis of CD8+, CD4+ and DN subsets.

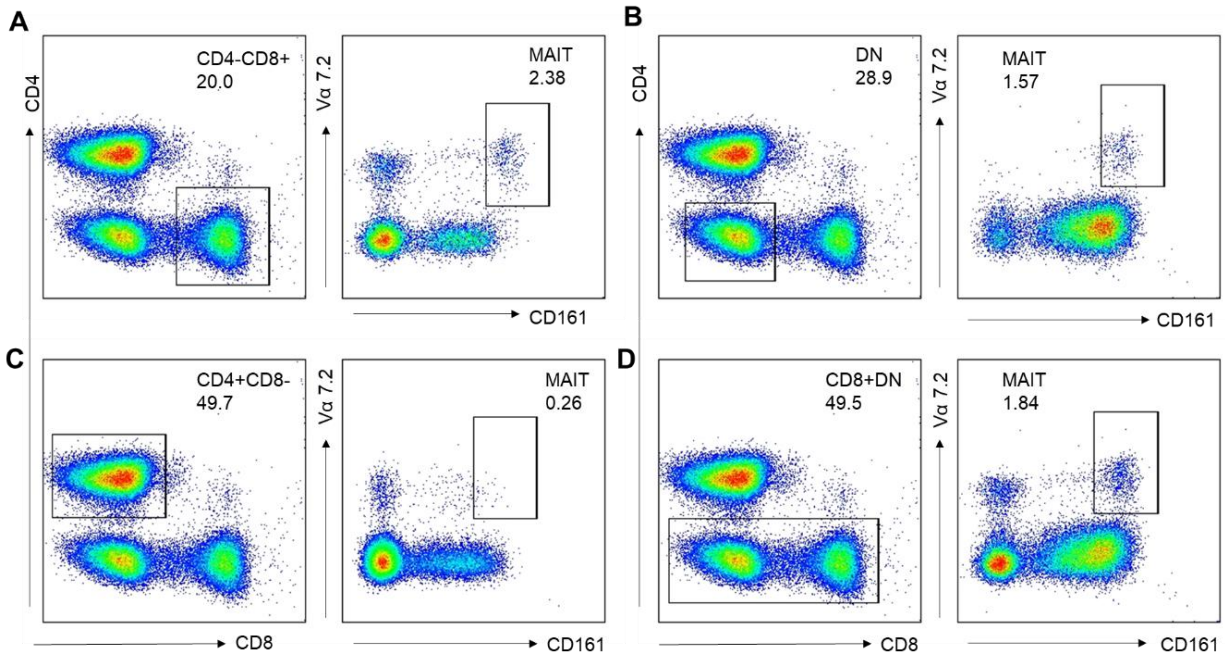

Figure 4.3: Gating strategy for MAIT cells (one representative sample shown). Representative flow cytometry dot plots show MAIT cells within the **(A)** CD4- CD8+ positive, **(B)** CD4-CD8-, **(C)** CD4+CD8-and **(D)** Combined CD8+ and DN within the T cell population, MAIT cells were identified as CD161++ Vα 7.2+.

4.3.4. MAIT cells and MAIT- IFN-γ expression are reduced in acute melioidosis patients who died compared to survivors

The frequency of MAIT cells in PBMC was analysed in acute melioidosis cases (PBMC collected during their first week of hospital admission). MAIT cell frequency and other properties including activation and presence of cytotoxic molecules was determined *ex-vivo* in unstimulated cells. To elucidate IFN-γ production by MAIT, cells were stimulated overnight (18 hours +/- 1 hour) with BP, *E. coli* and SEB. A significantly reduced (p=0.033) number of MAIT cells was found in the combined CD8+/DN T cell population from acute survived cases (median=2.5%, n=13) compared to died cases (median=1.4%, n=13) (Figure 4.4 A). A decreasing trend was also seen in the CD8+ and DN populations separately. IFN-γ expression by DN MAIT cells (Figure 4.4 C) and combined CD8+/DN MAIT cells (Figure 4.4 D-F) was significantly reduced (p=0.038 and 0.032 respectively) in acute melioidosis patients who died (n=13) compared to survivors (n=17). The median frequency of IFN-γ+ MAITs was 1.12% (survivors) versus 0.01% (died) in the DN population, and 0.85% (survivors) versus 0.01% (died) in the combined CD8+/DN population. The expression was also significantly stunted without stimulation (in media) and after *E.coli* and SEB stimulation in both compartments. Furthermore, the data indicates a trend for decreased IFN-γ production by CD8+ and CD4+ MAIT cells between the groups after BP and *E. coli* stimulation (Figure 4.4 B and E).

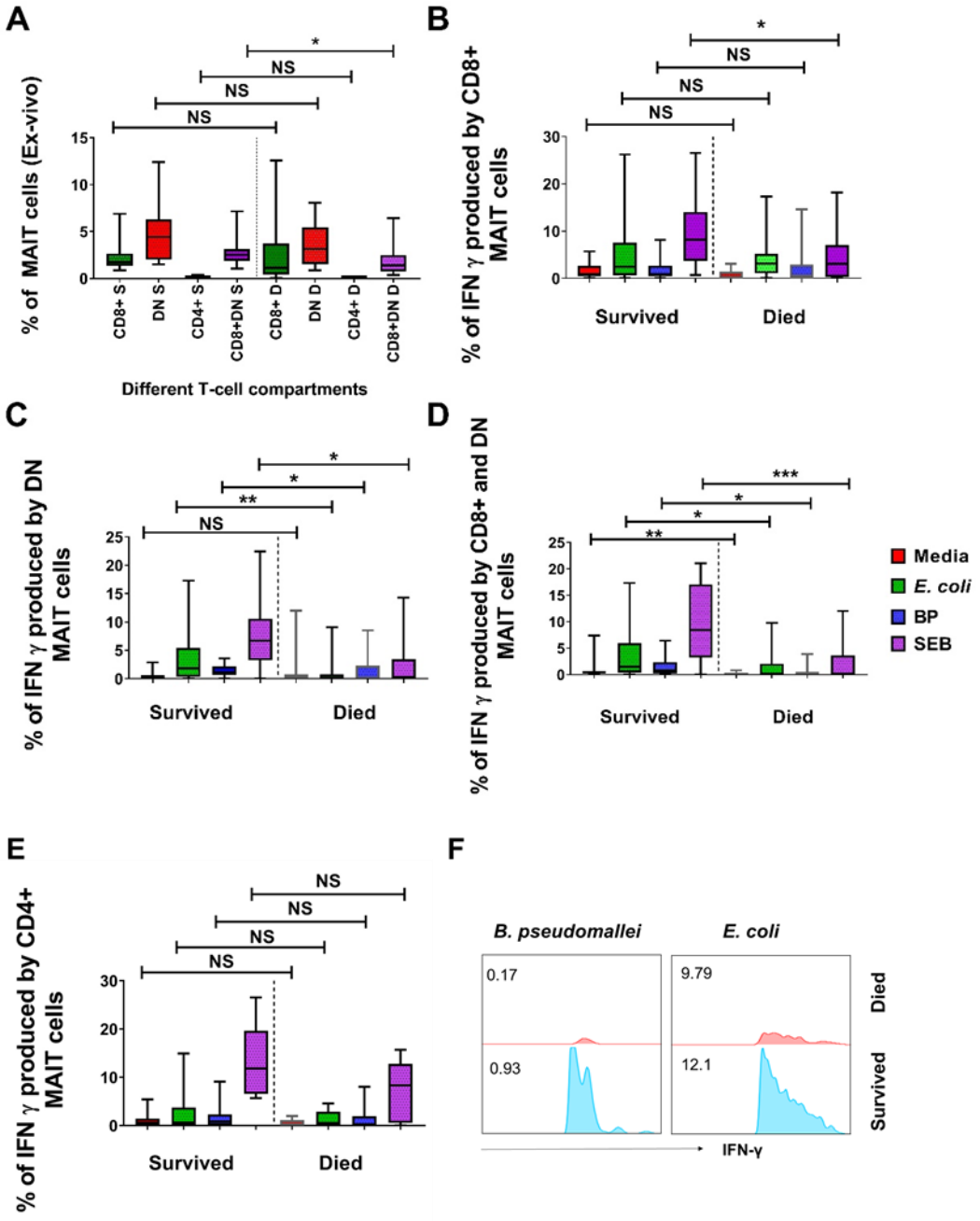

Figure 4.4: Comparison of MAIT cell frequency and IFN-γ production between acutely ill survivors and died melioidosis patients. A. The frequency of MAIT cell ex-vivo in CD8+, DN, CD4+ and combined CD8+DN T-cell population were compared (n=11-13 for survived and 12-13 for died) between survived (S) vs died (D). **B.** Percentage of IFN-γ production by CD4-CD8+ MAIT cells, **C.** CD4-CD8- MAIT cells, **D.** CD8+DN MAIT cells **E.** CD8-CD4+ MAIT cells. The comparison was performed between acute survived (n=13-17) and died (n=9-13) cases with (*E. coli*, BP and SEB) or without (R10; media) stimulation for 18 ±1 hours. **F.** Histogram showing comparison between one survived and one died case with IFN-γ production in BP and *E. coli*. Data is presented as box and whiskers plots with a line at the median. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05)

4.3.5. MAIT cells and IFN-γ production by MAIT cells are reduced in acute melioidosis patients compared to healthy endemic controls

Healthy subjects who were seronegative for BP by IHA (a serological test used to identify exposure to the organism, see Chapter 1) were studied alongside samples from acute patients (week 0) with culture-confirmed melioidosis. MAIT experiments were performed as described above (section 4.3.4).

A significant reduction ($p=0.049$) in the number of MAIT cells within the DN population was found (Figure 4.5 A) in acute melioidosis patients (median=4.1; n=25) compared to endemic healthy samples (median=6.6; n=17). A decreasing trend was also evident in the CD8+ (median=1.7% versus 1.6%) and combined CD8+/DN population (median=3.1% versus 2.1%) when comparing endemic controls and acute cases. The ability of MAIT cells to express IFN-γ was dramatically suppressed in acute melioidosis patients (n=28) compared to healthy controls (n=32), with a more than 8-fold decrease in median frequency within CD8+ (Figure 4.5 B) and DN (Figure 4.5 C) T cell populations ($p<0.0001$ for both). IFN-γ expression was also significantly suppressed (13- and 2-fold decrease in median frequency respectively) in combined CD8+/DN and CD4+T cell populations (Figure 4.5 D- F, $p<0.0001$ and $p<0.05$ respectively). Furthermore, IFN-γ expression was significantly stunted in the absence of stimulation in the combined CD8+/DN MAIT population and after *E.coli* and SEB stimulation in all of the MAIT cell compartments (except SEB for CD4+ MAIT, $p<0.0001$).

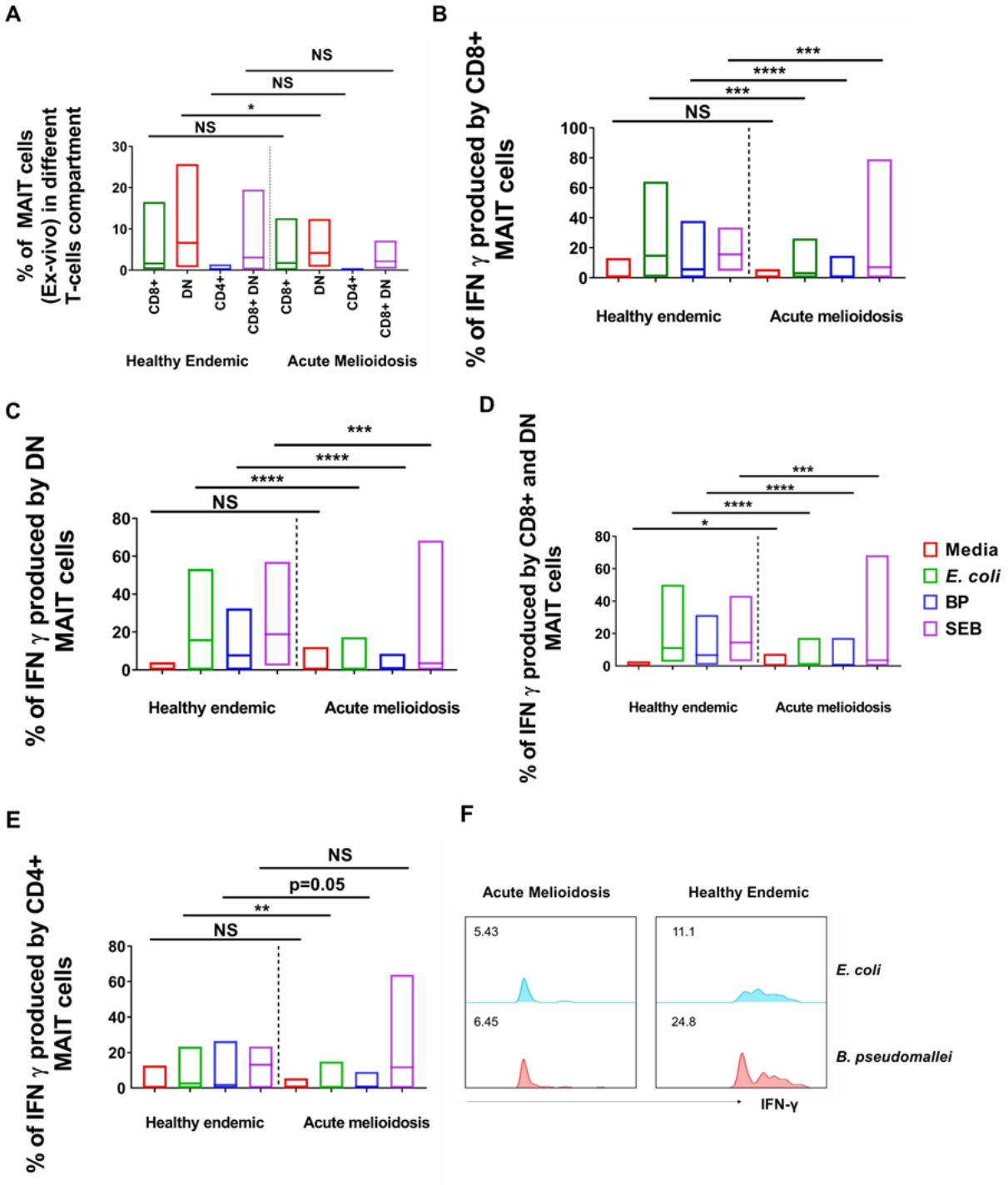

Figure 4.5. Comparison between healthy endemic and acute melioidosis samples in terms of frequency (%) of MAIT cells and MAIT induced IFN-γ expression. A. The frequency of MAIT cell ex-vivo in CD8+, DN, CD4+ and combined CD8+DN T-cell population were compared between healthy endemic (n=17-18) and acute cases (n=23-26). **B.** Percentage of IFN-γ production by CD4-CD8+ MAIT cells, **C.** CD4-CD8- MAIT cells, **D.** CD8+DN MAIT cells, **E.** CD8-CD4+ MAIT cells. The comparison was performed between acute (n=19-28) and endemic control (n=28-31) cases with (*E. coli*, BP and SEB) or without (R10; media) stimulation for 18 hours. **F.** Histogram showing comparison between an acute melioidosis case and a healthy endemic individual with IFN-γ production after BP and *E. coli* stimulation. Data is presented as floating bars with a line at the median. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

4.3.6. The activation marker CD69 is significantly higher in sepsis patients compared to different controls, without any difference in GzmB expression

The gating strategy to identify MAIT cells in different T cell compartments was the same as described in Figure 4.1 and 4.3. Gating for CD69 and GzmB is shown in Figure A-D for four different groups of patients (one individual from each group): acute melioidosis survived, acute melioidosis died, endemic healthy control and melioidosis recovered patient (week 52).

The activation marker CD69 was significantly higher (p=0.0374) in acute patients who died (median=54.9%, n=12) compared to survivors (median= 33.6%, n=11). It was also high (Figure 4.6 E) compared to recovered patients (median= 11.0%, n=21; p=0.0023) and healthy endemic controls (median=9.9%, n=12; p=0.0004). There was no significant difference in the cytotoxic effector molecule GzmB expression between the groups (Figure 4.6.F), although there was a trend towards higher expression in acute survived (median 20.4% versus 8, 7%) and recovered samples (18.2% versus 8.7%) compared to endemic healthy controls.

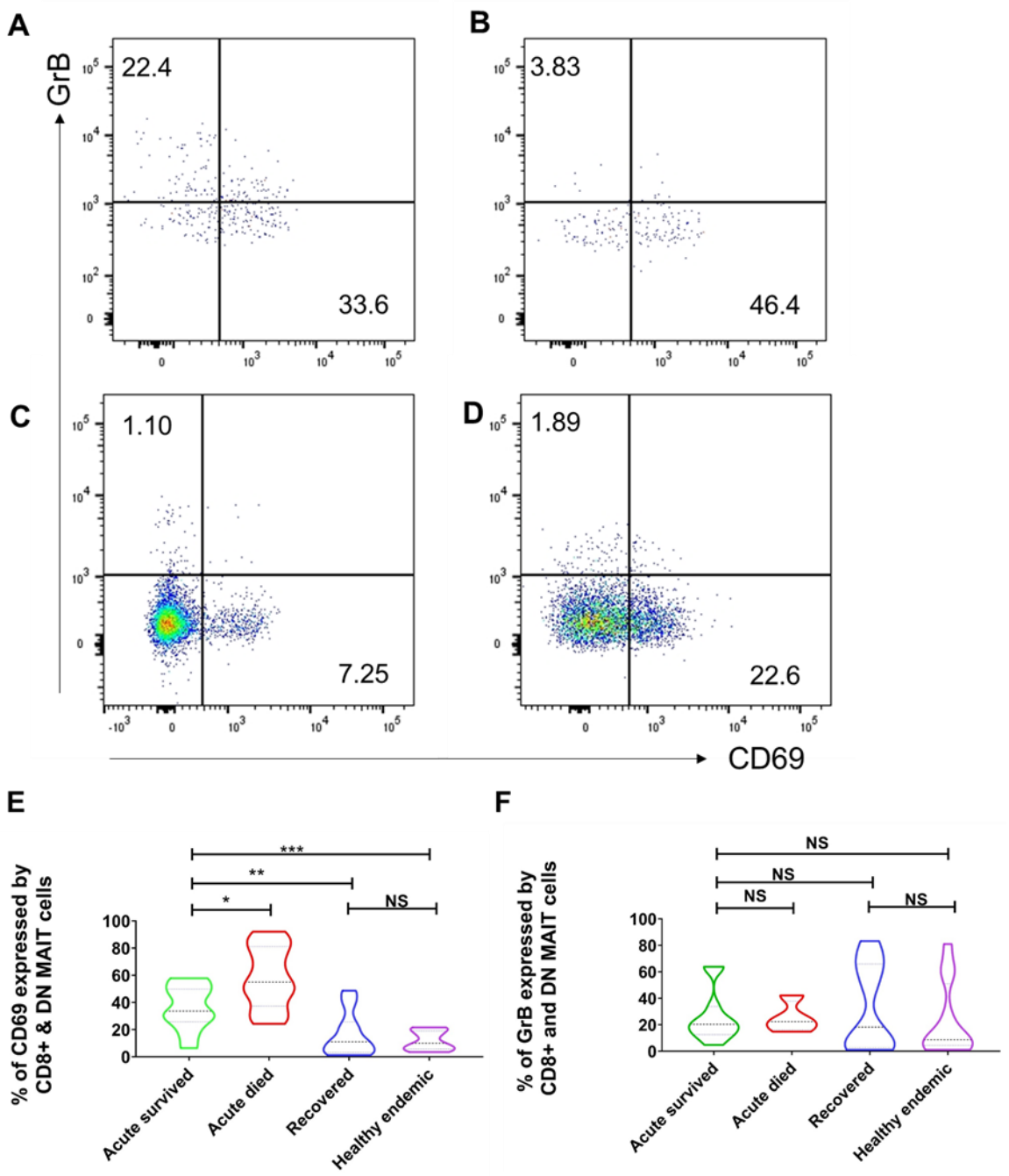

Figure 4.6. Comparison of activation marker CD69 and cytotoxic effector molecule Gr B between different melioidosis patient groups and controls (ex-vivo). Representative flow cytometry dot blots showing CD69 and GzmB expression on lymphocytes for each individual case of **A.** Acute melioidosis survived, **B.** Acute melioidosis died, **C.** Endemic healthy control and **D.** Melioidosis recovered patient. **E.** Violin plot (with a line at maximum, minimum and median) comparing percentage of CD69 between four groups. **F.** Percentage of Gr B between four different groups. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

4.3.7. IFN-γ expression upon BP stimulation is mediated by both MR-1 and cytokine dependent pathways, with greater predominance of cytokine-dependency

To determine whether activation of MAITs by BP is mediated through MR-1 or cytokine signalling, blocking experiments were performed using PBMC from healthy Oxford donors. In Figure 4.7 A, the contribution of MR-1 and IL-12+IL-18 to the production of IFN-γ inCD8+ MAITs was explored. Short (5 hours ± 1 hour) stimulation of PBMC with BP in the presence of THP1 as APC did not result in significant differences in terms of the pathway of activation. However, when they were co-cultured overnight (18 hours ± 1 hour), activation of MAITs were both MR-1 and cytokine-dependent, with more significant contribution (P=0.005) of the cytokine-mediated pathway. Blocking of MR-1 in conjunction with IL-12 + IL-18 almost ceased (p=0.001) the expression of IFN-γ. *E.coli* was used as a positive control, where the IFN-γ release was found to be significantly blocked by anti-MR-1 after 5 hours co-culture (Figure 4.7 B). Both the MR-1 and IL-12+IL-18 pathway significantly (p=0.005) contributed to IFN-γ expression after 18 hours of stimulation with *E. coli.* This finding is similar to the published literature from Prof Klenerman's lab (Kurioka et al., 2015, Ussher et al., 2014a).

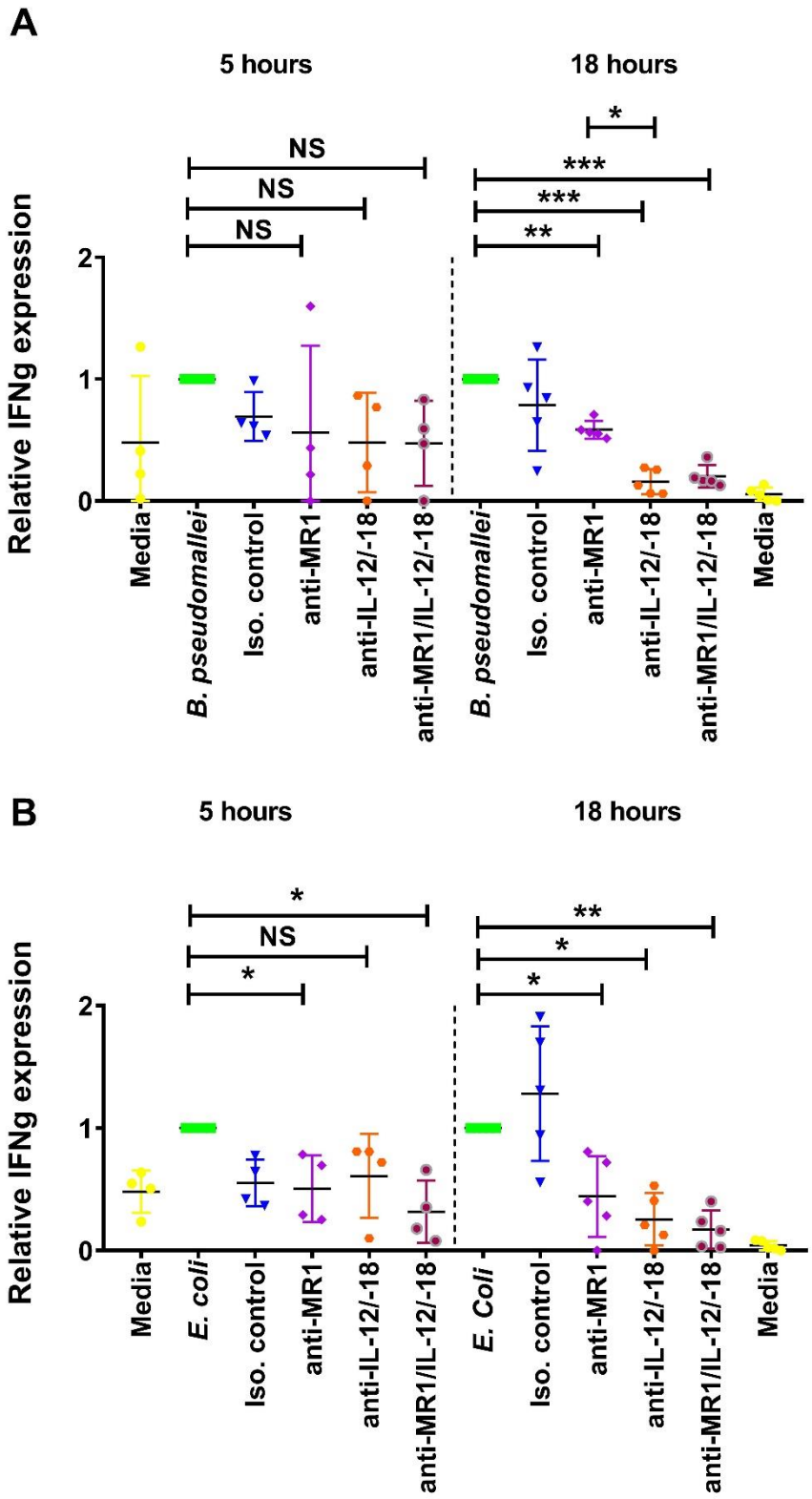

Figure 4.7. Blocking experiment of MAIT cell induced IFN-γ expression with BP and *E. coli* stimulation. IFN-γ production was compared after 5±1 (n=4) and 18±1 (n=5) hours incubation with **(A)** BP and **(B)** *E. coli*, THP-1 and PBMC, in the presence or absence of blocking antibodies against MR-1, IL-12, IL-18 and isotype control. Data were normalized to BP and *E. coli* with relative production of IFN-γ. Each symbol represents an individual sample. One-way ANOVA with Tukey's multiple comparison test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

4.4. Discussion

This is the first study to demonstrate and characterise the MAIT cell response to BP, and the first to study MAIT cells in acutely ill melioidosis patients. Initially, optimisation of the protocol (fig 4.1 A, B) to study MAIT cell function in response to BP in CD8+, DN and CD4+ T cells was performed. As a positive control, an optimisation experiment with *E. coli* was also carried out. We found MOI 50 and MOI 30 for BP and *E coli* respectively as the optimum concentration to use as a stimulant (fig 4.2). Separate staining with extracellular and intracellular markers was not found to be effective in the case of *E. coli*, though it worked well with BP (fig 4.1 C, D). Using extra and intracellular markers together as a cocktail (combined) functioned best in both the cases. One possible explanation for this is activation-mediated downregulation of surface markers, especially CD161 (Leeansyah et al., 2013).

We studied cells from acute melioidosis patients and compared fatal cases with survivors (fig 4.4). The role of MAIT cells in survival after acute infection has recently been studied in animals. In *Legionella longbeachae* pulmonary infection of mice, MAIT cells are demonstrated to be important for host defence (Wang et al., 2018). In Vα19i transgenic C57BL/6 mice (have high MAIT cell frequencies), the dynamic cytokine profile of MAIT cells were investigated 7 and 100 days after infection. IL-17A, IFN-γ and GM-CSF secretion was found to be significantly higher during acute infection compared to uninfected mice or after resolution (Wang et al., 2018). The effect was more pronounced in mice lacking CD4+ cells, and the activation was MR-1 dependent. Mice infected with a live strain of *Francisella tularensis* revealed robust activation of MAIT cells in an MR-1 dependent manner, with production of IFN-γ, TNF-α, and IL-17A to control bacterial growth (Meierovics et al., 2013). The MAIT

response to TB in a murine model showed mixed results. MAIT cells expressing CXCR3 and α4β1 were found to be recruited into the lungs and provided early protection in pulmonary TB infection (Sakala et al., 2015). However, in contrast, Kauffman *et al*. reported a poor response in TB-infected rhesus macaques, with low CD69, Gr B and Ki67 expression (Kauffman et al., 2018).

The MAIT response was found to be very important for survival against two strains of influenza-A virus in a murine model (Wilgenburg et al., 2018). MAIT cell-deficient MR-1 knock-out mice lost significant weight and died quickly compared to uninfected mice after severe (H1N1) influenza infection (Wilgenburg et al., 2018). Cytokine-dependent early activation with upregulation of CD25, CD69 and GzmB was seen during severe H1N1 infection.

I found acute melioidosis patients who survived had a higher number of MAIT cells compared to fatal cases, particularly in the combined CD8+/DN T cell population. The IFN-γ expression by MAIT cells was significantly stunted in acute patients who died compared to survivors after stimulation with either *E. coli*, BP or SEB. Interestingly, the decay was more pronounced in the DN MAIT population rather than in CD8+ T cells. DN subsets have been reported to exhibit higher apoptotic gene signatures and to be more prone to activation-induced apoptosis (Dias et al., 2018). In addition, it was shown that the DN MAIT cell population expressed a higher RORγt/T-bet ratio, and secreted fewer IFN-γ and more IL-17 than the CD8+ MAIT cell population, and these differences may explain our findings. The functional difference was also significant for unstimulated samples.

The majority of patients in the study presented with acute sepsis and overall, a very low frequency of MAIT cells. These findings coincide with the study of Grimaldi *et al.* where marked depletion of MAIT cell and other innate-like T lymphocytes was found

in acute non-streptococcal septic patients compared to healthy controls (Grimaldi et al., 2014). The MAIT cell response is well studied in patients with pulmonary TB, with similar findings. MAIT cell numbers were significantly lower in the peripheral blood of active pulmonary TB patients compared to healthy control and latent TB cases in both children and adults (Kwon et al., 2015, Malka-Ruimy et al., 2019). CD8+ MAIT cell induced IFN-γ expression was also significantly reduced during active mycobacterial infection (Kwon et al., 2015, Malka-Ruimy et al., 2019).

This work also revealed stunted IFN-γ secretion while comparing acute melioidosis patients with endemic healthy controls in each compartment of the MAIT cell population. This is further supported by Vorkas *et al*'s study where they found depressed IFN-γ response in TB contact participants compared to healthy control (Vorkas et al., 2018). Although *Mycobacterium* does not produce riboflavin or folate metabolites during their synthesis, it could still activate MAIT cells via the cytokine-dependent pathway (Kim and Oldham, 2019). A recently conducted *in-vitro* study infecting human blood with ten *Streptococcal pneumoniae* reference strains demonstrated significantly lower production of IFN-γ and higher activation of CD69 in MAIT cells compared to healthy controls (Kurioka et al., 2018). In acute bacterial infection, there is evidence of diminished functional capacity in the reduced MAIT cell population in the circulation (Wong et al., 2017). These functionally impaired cells express higher levels of the pro-apoptotic markers PD-1, PDL1, PDL2 and higher levels of inhibitory co-stimulating molecules (Jiang J, 2014, Wong et al., 2017). Jiang *et al*. also revealed that *in vitro* blockade of PD-1 can increase IFN-γ production from these impaired cells in patients with TB (Jiang J, 2014).

Researchers have also found very low circulating MAIT cells in acute cholecystitis (Kim et al., 2015), in a severe and fatal cystic fibrosis case (Pincikova et al., 2018) and in *Orientia tsutsugamushi* infection (Kang et al., 2016). Two potential

mechanisms that might explain this reduced MAIT cell number in acute infection or sepsis are apoptosis and the migration theory. Extensive apoptosis of both T and B-lymphocytes is the hallmark of sepsis (Grimaldi et al., 2014, Hotchkiss et al., 2009). In sepsis patients, massive apoptosis of immune effector cells occurs (Hotchkiss et al., 2009). In patients with active pulmonary TB, elevated expression of PD-1 was found to be associated with MAIT cell deficiency (Kwon et al., 2015). MAIT cells may also migrate into the site of infection resulting in lower levels in the circulation, although this mechanism has yet to be proven. Mixed results have been observed both in animal and human studies. Rapid accumulation of MAIT cells in mouse lungs has been described in the case of *Legionella* (Wang et al., 2018), *Francisella* (Meierovics et al., 2013) and influenza (Wilgenburg et al., 2018) infection. However, in rhesus macaques, very low numbers of MAIT cells were found in tuberculous granulomas (Kauffman et al., 2018). In humans, substantial numbers of MAIT cells have been found in TB-affected lungs and in ascitic fluid from tuberculous peritonitis patients (Gold et al., 2010, Le Bourhis et al., 2010). Another large case series showed diminished frequencies of MAIT cells in TB pleural effusions but not in ascitic fluids from patients with tuberculous peritonitis (Jiang J, 2014). In children with active pulmonary TB, MAIT cells also did not accumulate and proliferate in the lungs (Malka-Ruimy et al., 2019). It is therefore still unclear whether MAIT cell depletion in bacterial infection and sepsis is due to redistribution of cells into the organs of primary involvement. The reduced cellular response could also be due to a generalised immune failure as part of sepsis. Previous work in the melioidosis patient population used in Prof Dunachie's laboratory showed that BP-specific T cell responses were reduced in fatal cases, compared to survivors, but there was no significant difference between these groups in T cell response to a control panel of

viral T cell epitopes (Jenjaroen et al., 2015). This goes against the theory that lower BP specific cellular responses are solely a non-specific bystander effect of sepsis.

In this experiment, decreased MAIT cells frequency were seen in acute patients samples (*ex-vivo*) compared to endemic controls. Interestingly, this diminished MAIT cell frequency was significant for the DN population following BP stimulation, although not in *E. coli*. This may be due to divergence of transcription factors in response to stimuli between DN and CD8+ T cells, although both cell types possess the same developmental lineage (Dias et al., 2018). MAIT cell frequency and MAIT cell function (IFN-γ, TNF-α, GzmB) was reduced in response to *E. coli* in primary sclerosing cholangitis patients compared to healthy controls (von Seth et al., 2018). In my work, the activation marker CD69 was significantly higher in melioidosis patients (both survived and died) compared to different controls (recovered patients and healthy endemic). I also found higher GzmB expression in acutely ill and recovered melioidosis patients compared to endemic controls. In murine models robust activation and expression of CD25, CD69 and Gr B was found (5 days post-infection) in influenza-infected mice (Wilgenburg et al., 2018). However, another study performed in rhesus macaques reported little evidence of CD69 and Gzm B expression in tuberculous granuloma (Kauffman et al., 2018).

In-vitro Streptococcus pneumoniae infected human PBMC exhibit high activation of CD69 compared to controls (Kurioka et al., 2018). Human *ex-vivo* lung explant tissue infected with a clinical strain of *Haemophilus influenza* showed upregulation of GzmB and CD107a compared to healthy controls (Wallington et al., 2018). Higher activation of CD69 on MAIT cells was detected in the blood of children with active TB compared to healthy controls (Malka-Ruimy et al., 2019). In adults, upregulation of CD25 and Gzm B and downregulation of CD69 was observed in TB-exposed uninfected contacts compared to community controls (Vorkas et al., 2018) which also concurs

with my findings. PBMC from healthy individuals infected with *Clostridium difficile* infection also confirmed MR-1-dependent higher expression of CD69, GzmB and perforin compared to controls in a dose-dependent manner (Bernal et al., 2018). In acute viral infection (dengue, Zika and influenza), higher activation of MAIT cells (in terms of CD69, CD38 and HLA-DR) was observed (Paquin-Proulx et al., 2018, van Wilgenburg et al., 2016). Higher CD69 expression was also found in acute scrub typhus infection (Kang et al., 2016).

This work tracked the mode of inducing cytokine production, and demonstrated that IFN-γ release by MAIT cells in response to BP is both TCR- and cytokine-dependent, with more dependence on the cytokine-mediated pathway after long co-incubation (18 hours ± 1 hour). Blocking of MR-1 together with IL-12 and IL-18 completely ceased the expression of IFN-γ. *E. coli* was used as a control in this experiment and it has been found that early activation (5-hours±1 hour) was dependent on TCR, whereas both pathways mediated late activation. Others while experimenting with *E. coli* reported similar observations (Kurioka et al., 2015, Ussher et al., 2014a). Bernal *et al.* also revealed that *C. difficile*-induced IFN-γ expression is primarily MR-1 dependent, but cytokines play a role after long incubations (Bernal et al., 2018). Upon short incubation (5 hours ± 1 hour) with BP, no significant dependence on either pathway (TCR or cytokine) was observed. This suggests that MAIT cell activation and cytokine release could be a pathogen-specific phenomenon. Different organisms have different types of ligands, which are identified by APC through binding to their respective receptors, and trigger release of cytokines and other cytotoxic molecules. This mechanism also requires an optimum amount of time to present suitable amounts of MR-1-binding ligands to MAIT cells (Kurioka et al., 2015). It also depends on the nature of the antigen (live or fixed bacteria) used for stimulation (Kurioka et al., 2015). BP is known to be a resilient organism which can

survive within human phagocytic cells by escaping from the phagolysosome (Inglis et al., 2000). Therefore, partial or delayed expression of ligands to APC may occur, which subsequently affect the release of cytokines. On the other hand, BP possesses TLR- 2, 4 and 6, which can trigger cytokine stimulation without breaking down the bacteria (Wiersinga et al., 2018, Wiersinga et al., 2007a).

4.5. Limitations

Experiments with acute patient samples bring challenges. PBMCs were collected in Thailand from 2012 onwards, and later shipped to Oxford on dry ice. In some occasions, very low recovery of cells was achieved, precluding FACS staining and further analysis. The use of cryopreserved cells is a compromise because cells may undergo functional changes during freezing and thawing, in addition to cell loss. However, in order to undertake advanced immune analysis in patients with melioidosis, cryopreservation is essential because the catchment hospitals do not have the resources, skills, or in the case of the Thai hospital, physical space, for a flow cytometer. In addition, cryopreservation may allow for greater standardisation of sample processing, and experiments can be designed to allow comparison between groups and across different time points in one batch. The long duration of storage and maintenance of shipment temperature might be responsible for low recovery. Colleagues in Prof Dunachie's laboratory have undertaken a study comparing transport of PBMC from Thailand in liquid nitrogen versus dry ice (Rongkard *et al.*, *manuscript in preparation*). This work showed a modest improvement in cell recovery when cells were shipped in liquid nitrogen, but not at a level that justified the three-fold increase in cost. As alternatives to relying on stimulation of cryopreserved cells to characterise immune responses, our laboratory has pioneered transcriptomic

analysis in this population (Dunachie et al., 2017), and has also developed a whole blood stimulation assay for flow cytometry analysis of BP responses (Inthawong *et al.*, in preparation), but use of PBMC remains necessary to elucidate pathways. Use of tetramers for specific identification of MAIT cells could allow further power to study this cell group.

This work has identified further work including addressing the question of exhaustion or apoptosis of MAIT cells. It will be interesting to do future experiments with PD-1 and TIM-3 to look at exhaustion and Caspase 3 or Annexin IV for apoptosis. There are plans for our laboratory to take this forward.

4.6. Conclusions

In conclusion, this work has demonstrated an important role of MAIT cells in survival after BP infection. In particular, I found evidence of the importance of the DN MAIT cell compartment in the blood in host defence against acute BP infection. MAIT cells were found to be highly activated in acute patients compared to different control groups (melioidosis-recovered patients and endemic population), without any difference in Gr B expression. Vaccine strategies and therapeutics that target and boost MAIT cell function may be important for efficacy and improved survival.

5. Are MAIT cells important for host defence in people with or without DM against BP infection?

5.1. Introduction

DM is a major non-communicable disease (NCD) and a serious health problem currently affecting 425 million people worldwide(International Diabetes Federation, 2017). 12% of the global health expenditure is spent on DM, and 1 in every 11 adults (1 in 5 adults in South East Asia) worldwide has DM (International Diabetes Federation, 2017). At present, three-quarters of people with DM live in LMIC (International Diabetes Federation, 2017). People with DM have increased risk of infection and / or worse outcomes with bacteria (particularly Gram-negative and intracellular), virus, fungi and some parasites (Dunachie and Chamnan, 2018). For TB, DM is associated with a two- to four-fold increased risk of active TB (Al-Rifai et al., 2017), poorer treatment outcomes, treatment failure and death (Workneh et al., 2016). DM patients were more symptomatic than non-DM after two months of anti-TB treatment, and there was increased death amongst TB DM patients during the period of treatment (Workneh et al., 2016). DM is also an established risk factor (three-fold risk) for *Salmonella enteritidis* (Telzak et al., 1991), *Klebsiella pneumoniae* (Luo et al., 2016, Yang et al., 2004) and invasive *Staphylococcus aureus* (Bassetti et al., 2012) infection. However, the highest risk association (12 fold) is revealed in people with DM in Northern Australia and Thailand for BP infection (Currie et al., 2004, Limmathurotsakul et al., 2010).

In endemic areas, more than 50% of melioidosis cases have DM (Currie et al., 2010, Limmathurotsakul et al., 2006). This signifies a unique relationship between DM and

melioidosis. CMI to BP is associated with IFN-γ, IL-12, IL-18 and TNF in different studies performed in animal models (Meierovics et al., 2013, Wang et al., 2018) and humans (Koh et al., 2013, Tippayawat et al., 2009). Our laboratory demonstrated a strong relationship between CD4+ and CD8+ T cell responses to the bacteria and mortality (Jenjaroen et al., 2015). MAIT cells are an innate-like T cell subpopulation, and have both innate and adaptive immune effects (Howson et al., 2018). There is a lack of knowledge on cellular immune responses in melioidosis patients with or without DM. The role of MAIT cell has never previously been explored in melioidosis patients with DM or in the endemic population with DM. In this chapter, we hypothesized that the IFN-γ secretion by MAIT cells would be lower in people with DM compared to non-DM. We also hypothesised that there would be higher MAIT cell activation, effector function and exhaustion in people with DM.

5.2. Aims

A. To identify the phenotype, activation and function of MAIT cell amongst the current population (DM versus non-DM) in Ubon Ratchathani, Thailand, a melioidosis-endemic region.

B. To compare the phenotype, activation and function of MAIT cells amongst recovered (week 52) melioidosis patients with or without DM.

C. To explore MAIT cell exhaustion in the otherwise healthy endemic control population, comparing between DM and non-DM.

5.3. Results

5.3.1. IFN-γ release by MAIT cells is reduced in people with DM compared to non-DM amongst the endemic population.

Healthy subjects (apart from some having DM) who were seronegative for BP by IHA were studied. MAIT experiments were performed *ex-vivo* (without stimulation), and to elucidate the IFN-γ production, cells were stimulated overnight (18 hours ± 1 hour) with BP, *E. coli* and SEB. Gating was performed on the lymphocyte and T cell populations as shown in Figure 4.1.

No significant difference in the number of MAIT cells was found (Figure 5.1 A) in a healthy endemic population with (n=15) or without DM (n=14-15), although a trend to lower numbers was noted amongst the DM population in all the MAIT cell compartments. The median frequency for DM versus non-DM participants was 2.5% versus 1.3% in the CD8+ population, 6.9% versus 4.2% in the DN and 3.8% versus 2.2% in the combined population respectively.

The ability of MAIT cells to express IFN-γ was significantly suppressed in DM control subjects (n=13) compared to non-DM healthy controls (n=15), with a more than 4-fold and 5-fold decrease in median frequency compared to non-DM in CD8+ (Figure 5.1 B) and DN (Figure 5.1 C) T cell populations respectively after BP stimulation for 18 hours. There was a non-significant trend to lower IFN-γ expression in CD4+ T cells (Figure 5.1 E). IFN-γ expression was significantly suppressed (more than 2-fold decrease in median frequency) in the combined (CD8+& DN) T cell population (Figure 5.1 D-F). Expression was also significantly stunted after *E.coli* stimulation in all the MAIT cell compartments except for CD4+ MAIT. No significant difference was noted in media and after SEB stimulation.

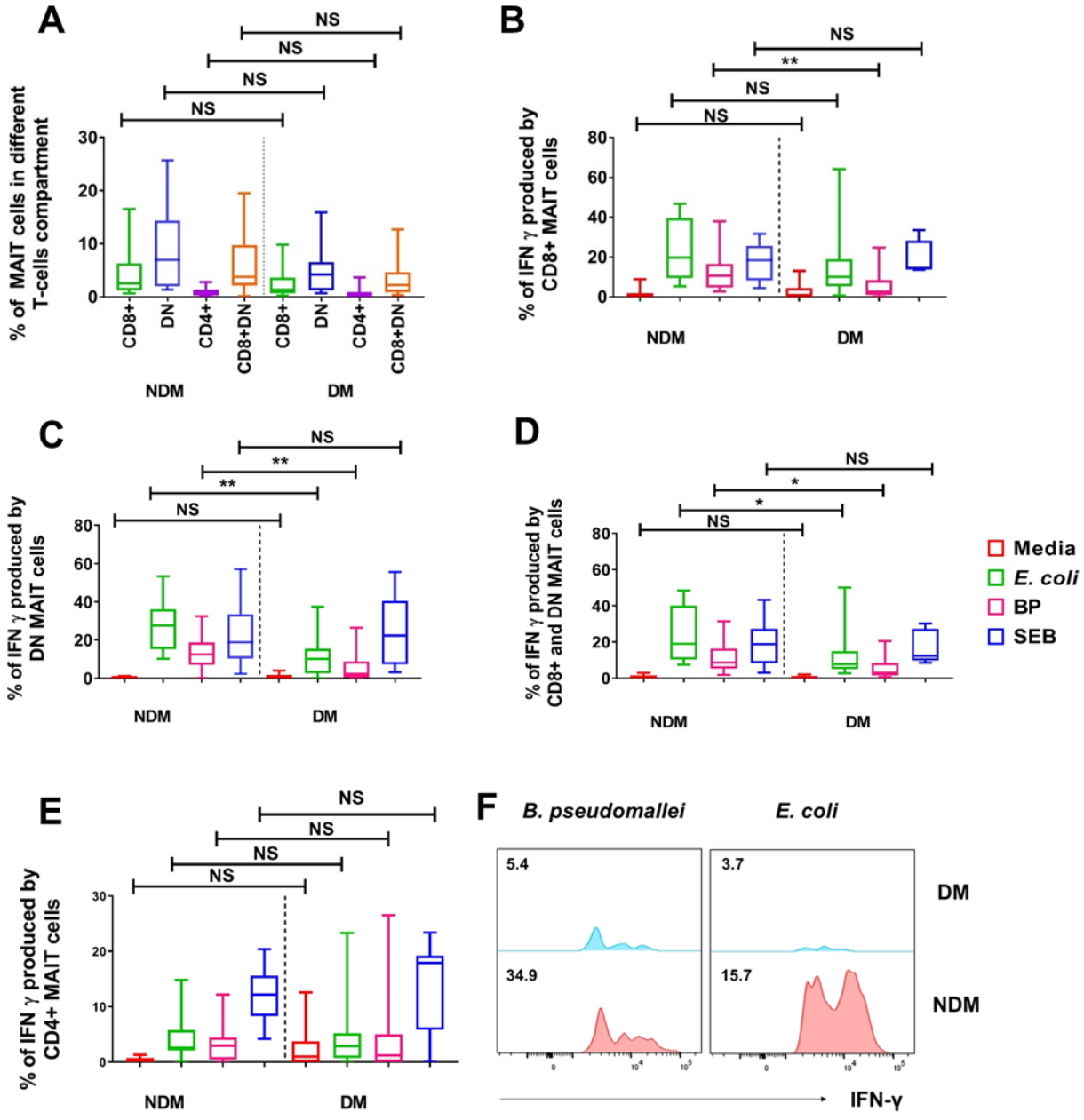

Figure 5.1. Comparison between DM versus non-DM amongst otherwise healthy melioidosis endemic samples in terms of frequency (%) of MAIT cells and IFN-γ expression. A. The frequency of MAIT cells *ex-vivo* in CD8+, DN, CD4+ and the combined CD8+DN T-cell population was compared between endemic DM (n=15) and non-DM participants (n=14-15). **B.** Percentage of IFN-γ production by CD4-CD8+ MAIT cells, **C.** CD4-CD8- MAIT cells, **D.** CD8+DN MAIT cells **E.** CD8-CD4+ MAIT cells. The comparison was performed between DM and Non-DM endemic participants with (*E. coli*, BP and SEB) or without (R10; media) stimulation for 18 hours. **F.** Histogram showing a comparison between a healthy endemic case of DM and Non-DM individual with IFN-γ production after BP and *E. coli* stimulation. Data are presented as floating bars with a line at the median. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

5.3.2. No difference was found in the frequency of MAIT cells and MAIT-IFN-γ expression in recovered melioidosis patients comparing between DM and non-DM.

The frequency of MAIT cells in peripheral blood (PBMC) was analysed in recovered melioidosis cases (PBMC collected 52 weeks after infection) in a follow-up clinic. For MAIT cell data, the experiment was performed *ex-vivo* (without stimulation), and to elucidate the MAIT- IFN-γ production, cells were stimulated overnight (18 hours ± 1 hour) with BP, *E. coli* and SEB. Gating was performed on the lymphocyte and T cell populations as shown in Figure 4.1.No significant reductions in the frequency of MAIT cells were found (Figure 5.2 A) in healthy recovered patients with or without DM, although a decreasing trend was noted amongst the DM population in the DN MAIT cell compartments. The median frequency in percentage was 5.6% versus 6.7% in DM (n=15) and non-DM (n=12) respectively.

No significant difference was noticed between DM and non-DM amongst recovered melioidosis patients in terms of IFN-γ secretion by MAIT cells. A trend of higher expression was noted in patients with DM in all the compartments of MAIT cells (Figure 5.2 B-F). The median frequency in percentage for DM (n=9-12) and non-DM (n=5-10) following BP stimulation (18 hours) was 9.4% versus 6.1% for CD8+, 11.1% versus 5.2% for DN, 3.2% versus 5.5% for CD4+ and 8.9% versus 6.8% for CD8+ & DN combined MAIT cell population respectively. The trend was similar after *E. coli* stimulation but not in the case of SEB stimulation.

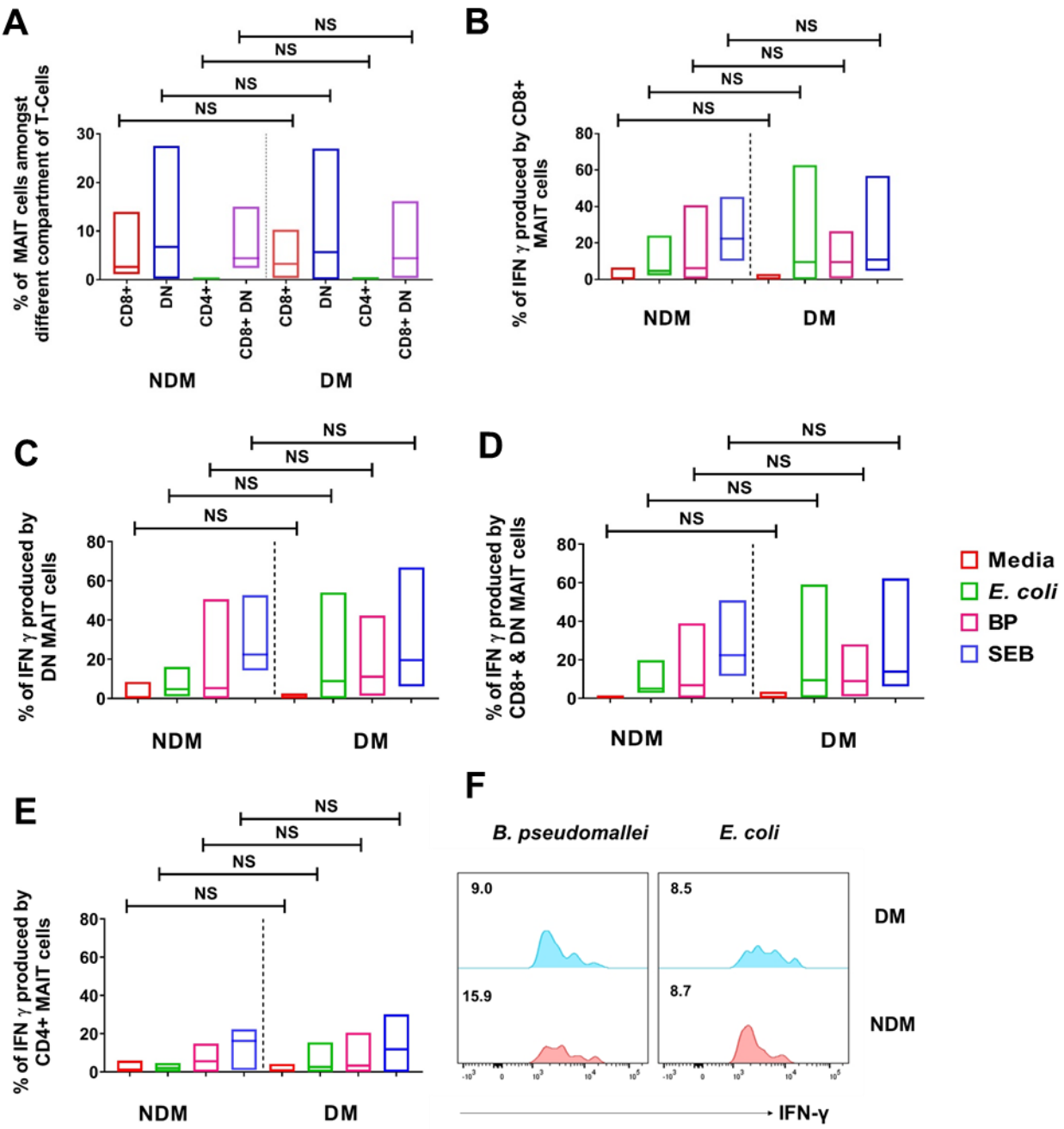

Figure 5.2: Comparison of MAIT cell frequency and IFN-γ production between recovered patients with or without DM. A. The frequency of MAIT cell *ex-vivo* in CD8+, DN, CD4+ and combined CD8+DN T-cell population were compared in recovered patients (52 weeks post infection) between Non-DM (n=5-12) vs DM (n=8-15). **B.** Percentage of IFN-γ production by CD4-CD8+ MAIT cells, **C.** CD4-CD8- MAIT cells, **D.** CD8+DN MAIT cells, **E.** CD8-CD4+ MAIT cells. The comparison was performed between melioidosis patients with DM (n=9-12) and without (n=5-10) with (*E. coli*, BP and SEB) or without (R10; media) stimulation (18 hours ± 1 hour). **F.** Histogram showing a comparison between one patient with DM and one without with IFN-γ production in BP and *E. coli*. Data are presented as a floating bar with a line at the median. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

5.3.3. No difference was revealed for the expression of activation marker CD69 and cytotoxic effector molecule Gzm B expression between DM and non-DM populations

Healthy participants living in Ubon Ratchathani (a melioidosis-endemic zone in Thailand) and recovered melioidosis patients' PBMC were used for the experiment (*ex-vivo*). The gating strategy to identify MAIT cells in different T cell compartments was the same as described in Figure 4.1 and 4.6. No significant difference was found for the expression of activation marker CD69 in total CD4+ T cell, CD8+ T cell and MAIT cell population (CD8+ & DN combined), although there was a trend towards a higher percentage of expression in endemic participants with DM compared to non-DM. The median frequency of CD4+T cells (Figure 5.3 A) and CD8+ T cells (Figure 5.3 B) expressing CD69 amongst non-DM (n=8) and DM (n=8) endemic healthy participants was 6.0% versus 8.2% and 5.9% versus 6.5% respectively. The median percentage of CD69 expression was 8.9% versus 11.2% by MAIT cells (Figure 5.3 C) between the groups (n=7 and 6). No difference was found in terms of activation (Figure 5.3 E) in non-DM (19.6%) group compared to DM (10.3%) amongst melioidosis recovered patients.

There was no significant difference in the cytotoxic effector molecule GzmB expression between the groups (Figure 5.3.D and F) for endemic control and recovered patients, although there was a trend to higher expression in patients with DM compared to non-DM for both endemic and recovered patients. The median frequency was 5.4% versus 8.6% amongst endemic participants and 18.0% versus 30.9% amongst recovered patients in non-DM and DM respectively.

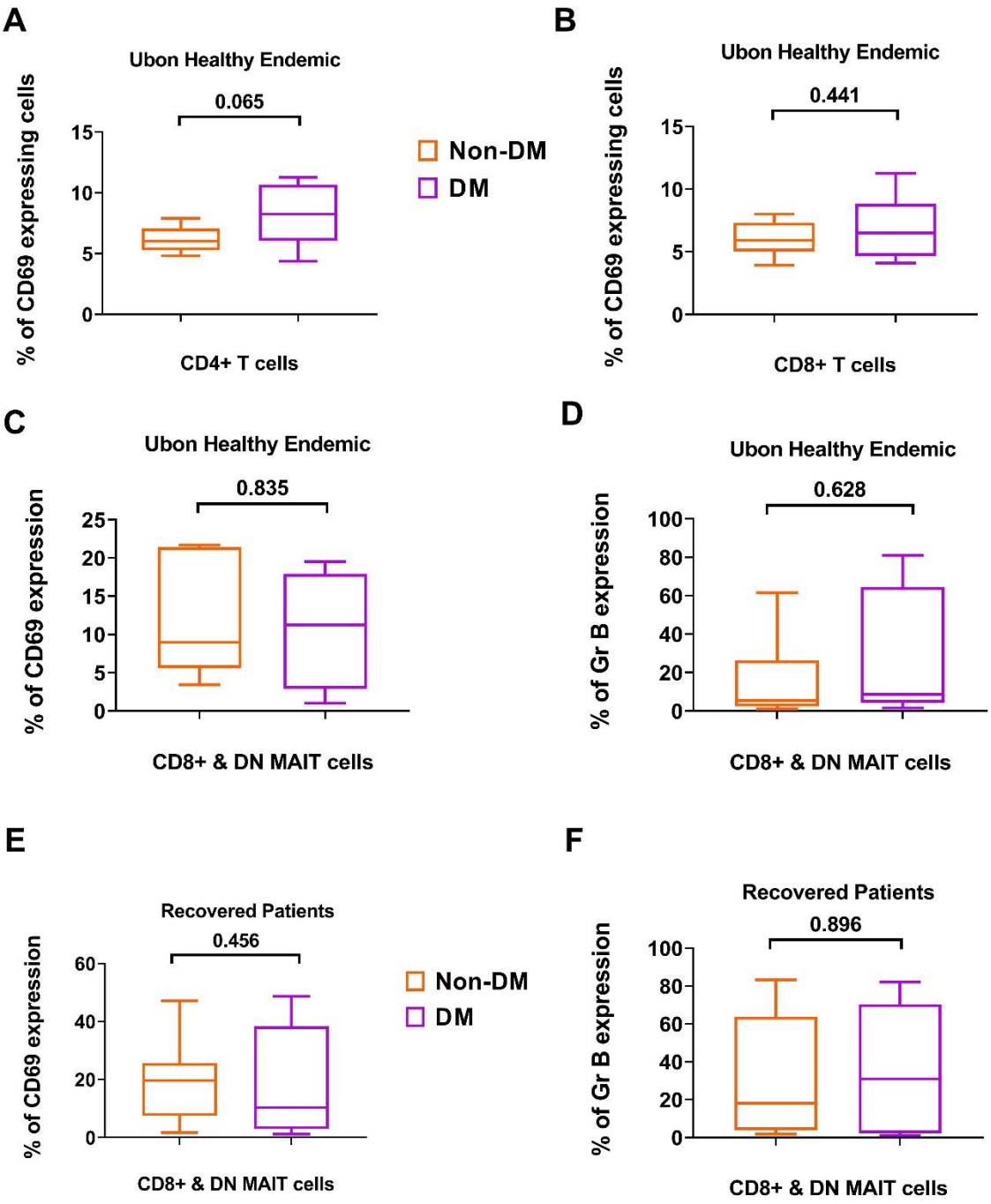

Figure 5.3. Comparison of expression of the activation marker CD69 and cytotoxic effector molecule GzmB between DM (n=6-11) and non-DM (n=7-9) in the melioidosis-endemic population and recovered patients (*ex-vivo*). A. Percentage of CD69 expressing CD4+ T cells, **B.** Percentage of CD69 expressing CD8+ T cells **C.** Percentage of CD69 expression by CD8+ & DN MAIT cells, **D.** Percentage of GzmB expression by CD8+ & DN MAIT cells, **E.** Percentage of CD69 expressed by CD8+ & DN MAIT cells. **F.** Percentage of GzmB expressed by CD8+ & DN MAIT cells. Figure A to D represent endemic participants and E-F melioidosis recovered patients (52 weeks post-infection). Data are presented as box and whiskers plots with a line at the median. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

5.3.4. There was no difference between DM and non-DM groups amongst the endemic population in terms of exhaustion marker PD-1 and TIM-3 expression.

The gating strategy to identify MAIT cells in different T cell compartments was the same as described in Figure 4.1 and 4.3. Gating for PD-1 and TIM-3 is shown in Figure A and B in a representative sample from each of the two different groups of the endemic population: DM and non-DM.

No significant difference was noted for the expression of PD-1 and TIM-3 between DM and non-DM amongst different T cell subpopulations. Percentage of CD4+ T cells (Figure 5.4 C) and CD8+ T cells (Figure 5.4 D) expressing PD-1 was 0.5% versus 0.8% and 0.2% versus 0.3% in DM versus non-DM respectively (n=8). In CD8+ & DN MAIT cells (Figure 5.4 E), the percentage of PD-1 expression was 87.6% versus 89.3% between the groups (n=6). Higher expression of TIM-3 was noted in people with non-DM compared to DM (61.8% versus 34.9%) which did not reach statistical significance (Figure 5.4 F).

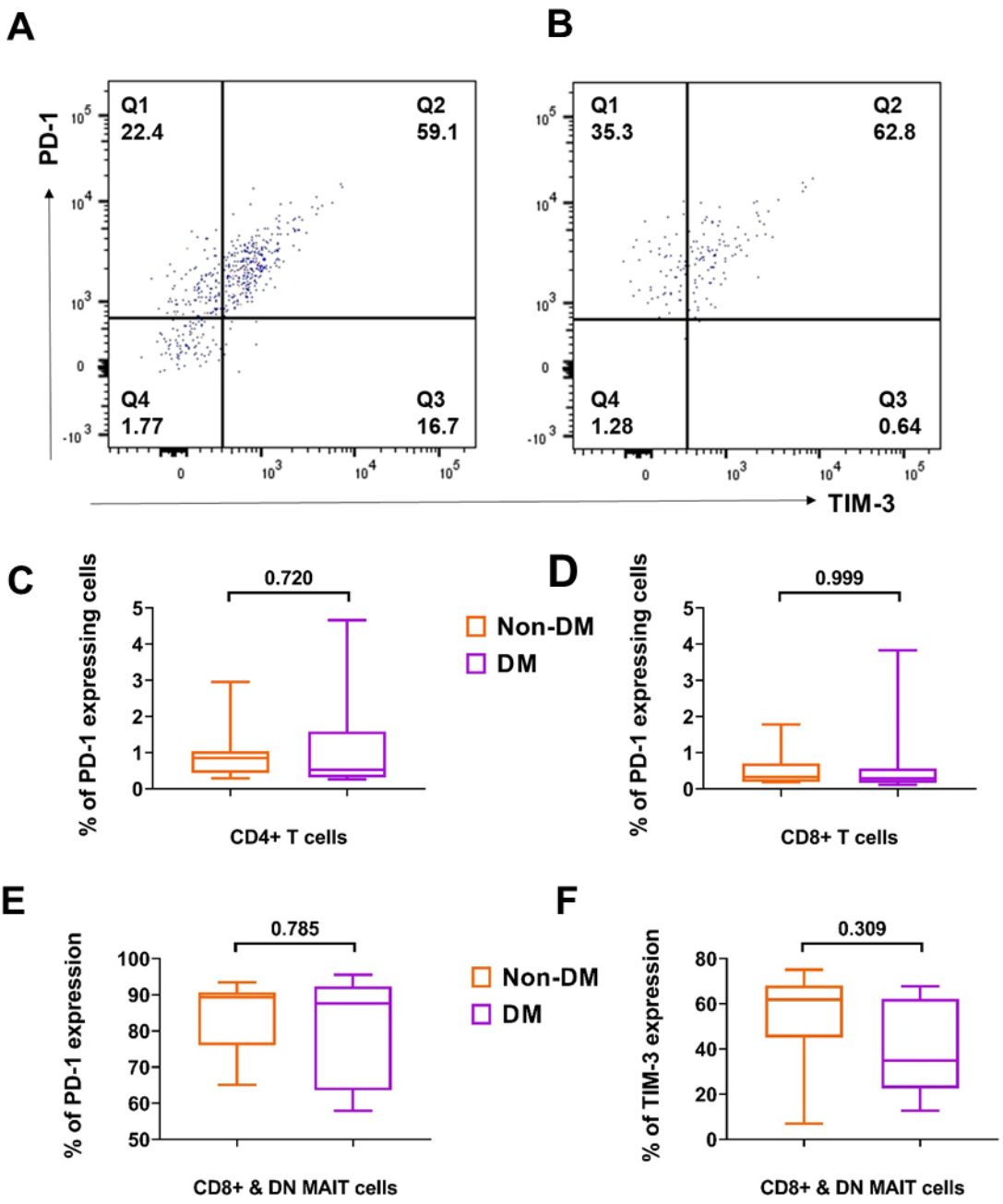

Figure 5.4 Comparison of exhaustion marker PD-1 and TIM-3 with (n=6-8) or without (n=6-8) DM amongst endemic controls (*ex-vivo*). Representative flow cytometry dot plots showing PD-1 and TIM-3 expression on lymphocytes for each individual case of **A.** Healthy endemic with non-DM, **B.** Healthy endemic with DM. **C.** Percentage of PD-1 expressing CD4+ T cells, **D**. Percentage of PD-1 expressing CD8+ T cells, **E.** Percentage of PD-1 expression by CD8+ & DN MAIT cells, **F.** Percentage of TIM-3 expressed by CD8+ & DN MAIT cells. Data are presented as box and whiskers plots with a line at the median. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

5.4. Discussion

The role of MAIT cells in immune-mediated diseases such as Type 2 DM (Magalhaes et al., 2015b), rheumatoid arthritis (Chiba et al., 2012), SLE (Cho et al., 2014) and others has drawn much interest in recent years. This is because of a range of interesting factors, including the variation in distribution of MAIT cells in different tissues, the presence of an effector memory phenotype for MAIT cells, and their pro-inflammatory profile (Hinks, 2016). I examined healthy subjects with and without DM from a melioidosis-endemic region in Thailand who were seronegative for BP. The effect of DM status on MAIT cell frequency and responsiveness to bacterial stimulation was explored. In this experiment, the melioidosis patients and participants with DM had not undergone formal typing of DM, but were believed to be predominantly Type 2 due to age of onset and not requiring insulin from the start of their DM diagnosis. Amongst the endemic control cohort, we did not find any significant difference between people with and without DM in terms of MAIT cell frequency, although a decreasing trend was noted in people with DM in all MAIT cell populations. Our findings are similar to the first such experiment performed in 2015, when Magalhaes *et al*. also found significantly reduced numbers of circulating MAIT cells in the blood and AT of Type 2 DM patients, and in severely obese people compared to healthy controls (Magalhaes et al., 2015a, Magalhaes et al., 2015b). Carolan *et al.* also revealed dysregulation of MAIT cells and a positive correlation between MAIT cell dysregulation and the severity of insulin resistance (IR; type 2 DM) (Carolan et al., 2015). They used a homeostasis model for the assessment of IR by calculating fasting plasma insulin (μU/l) × fasting glucose (mmol/l) divided by 22.5 (basal insulin level in blood). The cut-off was set 3.1 and any value more than that was considered as IR (Carolan et al., 2015). Both adult and child obese participants

were found to have increased IL-17+ MAIT cell frequency in AT and increased IR compared to healthy control. They also observed a reduced number of MAIT cells in peripheral circulation, increased accumulation in ATs, and altered cytokine production (reduced IL-10 and increased IL-17) in both adults and children with obesity (Carolan et al., 2015). Furthermore, MAIT cells from Type 2 DM patients were found to secrete higher levels of IL-17 when stimulated *in vitro* with MAIT cell ligand (Magalhaes et al., 2015a). This diminished frequency of MAIT cell in Type 2 DM could be due to increased production of IL-17. IL-17 recruits neutrophils and drives more inflammation to the AT (Carolan et al., 2015). When MAIT cells die after inflammation, recruitment of further MAIT cells from the circulation may contribute to lower MAIT cell frequencies in the peripheral blood. Therefore, the reduced frequency of MAIT cells may represent a redistribution rather than a reduction in total body MAIT, and this was confirmed by tissue sampling in adults (Carolan et al., 2015).

In my experiment, I found significantly decreased production of IFN-γ from MAIT cells in the melioidosis-endemic DM population compared to non-DM with both BP and *E. coli* post-stimulation. Our findings concurred with previous studies. In a murine model, impaired tissue cytokine (fold change in mRNA expression) expression (IFN-γ, TNF-α, IL-12, IL-6 and IL-1β) was found in diabetic mice compared to non-diabetic littermates after 24 and 72 hours of BP stimulation (Hodgson et al., 2015, Hodgson et al., 2013). Melioidosis patients with type 2 DM had lower plasma IFN-γ and higher pro-inflammatory cytokine IL-10, which is related to poor bacterial killing (Kessler et al., 2017). Poorly controlled DM patients infected with BP or MTB showed impaired IL-12p70 and IFN-γ secretion compared to control, which was also associated with poor bacterial killing (Tan et al., 2012). My findings are consistent with another study, where low IFN-γ and TNF-α production by MAIT cells was found when comparing

healthy Type 2 DM versus non-DM participants (Magalhaes et al., 2015b). The authors suggested that this reduced functional response could be due to activation-induced cell death. Upregulation of CD25 and CD69 expression (early activation marker of CD8 T cells) has been reported for MAIT cells from Type 2 diabetic patients compared to controls (Magalhaes et al., 2015b). I did not notice any significant change in CD69 expression of CD4+ T cells, CD8+ T cells and MAIT cells amongst our diabetic healthy endemic controls. MAIT cells in AT displayed higher expression of Ki67, which further suggests local recruitment of this cell into tissue (Magalhaes et al., 2015a). In metabolic diseases like DM, gut microbiota composition and gut permeability can also play an important role in MAIT cell activation (Magalhaes et al., 2015b), and it would be very interesting in future studies of this population to examine temporal changes in the microbiota in acute melioidosis and recovery with MAIT cell functionality.

A previous study by our laboratory revealed a non-significant trend towards lower *ex-vivo* IFN-γ response by T cells in DM survivors of melioidosis one year later compared to non-DM (Jenjaroen et al., 2015). I could not find any significant difference in MAIT-IFN-γ secretion amongst recovered diabetic melioidosis patients. However, a recent report from our laboratory found a significant predominance of γδ T cell-derived IFN-γ secretion was identified in the recovered DM group compared to non-DM (Kronsteiner et al., 2019). Higher expression of the cytotoxic effector molecule GzmB was noted in the diabetic group compared to non-diabetic in both healthy endemic and recovered patients. This is similar to another study where higher expression of GzmB was revealed in Type 2 DM participants compared to healthy controls (Magalhaes et al., 2015b). This is probably because DM is a pro-inflammatory state, and different metabolic mediators such as C-reactive protein, IL-6 might play an active role.

We further looked at why MAIT cells from the endemic DM population are less capable of secreting IFN-γ. No difference was noted for the expression of exhaustion marker PD-1 and TIM-3 between the groups in endemic healthy participants. This requires further experiments to seek a logical explanation.

5.5 Limitations

The sample size for the activation, phenotype and exhaustion experiment was small, due to the limited number of cells in the archive and limited time. Further work to address the mechanism of reduced functionality of MAIT cells in DM would be worthwhile, and are planned in the laboratory. It will be interesting to do future experiments with caspase 3 or Annexin IV for apoptosis. It would also be interesting to look at the subjects' body mass index (BMI) to compare between Type-2 DM with or without obesity. This is because MAIT cells have been found to be dramatically decreased in severe obesity along with higher activation in AT (Magalhaes et al., 2015b). Interestingly the number of MAIT cells and their functional response was found to be restored after bariatric surgery because of decrease circulating level of adiponectin (Magalhaes et al., 2015b). Unfortunately, although the study's CRF requested patient weight and height, this was only available for around half the cohort, and anecdotally the nurses said they did not weight the very heavy and / or unwell patients, so analysis where the BMI was available was felt to be too biased a representation of the cohort. The difference of gut microbiome composition, level of gut permeability, the presence of bacterial metabolites between DM versus non-DM would also be interesting to explore for future research.

As discussed previously, studying MAIT cells in the peripheral blood may not represent the body's entire MAIT cell population in terms of numbers and

functionality, and shifting of MAIT cells to AT may be particularly important in DM and obesity. More invasive studies with biopsy of AT would be required to address this.

5.6. Conclusions

In conclusion, MAIT cells were found to have a significantly lower functional response to BP in the melioidosis-endemic DM population compared to non-DM. The diabetic population also showed a non-significant trend to higher activation and higher cytotoxic molecule expression compared to non-DM. No evidence of MAIT cell exhaustion was demonstrated between the groups. People with DM appear to have impaired MAIT cell response to this bacterium, and development of therapies to boost MAIT cell numbers and / or function could improve the vulnerability of people with DM to infection.

6. MAIT cells in acute infection with different Gram-negative bacteria

6.1 Introduction

Bacterial infections are the commonest cause of adult sepsis in both HIC and LMIC. A range of bacteria are responsible for this. However, the majority of deaths in sepsis are due to Gram-negative bacteria (Daniels et al., 2018, Deen et al., 2012). In South and Southeast Asia, almost two-thirds of bacterial sepsis in adults are due to Gram-negative bacteria (Chatterjee et al., 2017, Deen et al., 2012, Limmathurotsakul, 2017). Gram-negative intracellular bacteria that synthesize riboflavin precursors during their metabolism can activate MAIT cells (Le Bourhis et al., 2010). Studies in mouse models suggest that MAIT cells could be crucial for survival (impaired pathogen clearance and death in MR1 deficient mice) after infection with Gram-negative bacteria (Georgel et al., 2011, Gold et al., 2010). In a human study enrolling severe sepsis and septic shock patients, MAIT cells were lower in number in this group compared to healthy controls (Grimaldi et al., 2014). The frequency of MAIT cells was also associated with the severity of sepsis.

No further studies have ever been reported evaluating the role of MAIT cells in patients infected with Gram-negative bacteria, and comparing MAIT cell responses to stimulation with the same bacterial species as each patient's pathogen is novel to our knowledge. In this context, I evaluated the role of MAIT cells in different common Gram-negative bacterial infections amongst culture-confirmed cases enrolled from fever cohorts in Bangladesh (Chapter 2.1) and Thailand. In this chapter, I hypothesize that IFN-γ secretion by MAIT cells will be lower in acute febrile patients infected with Gram-negative bacteria compared to healthy endemic controls. We also hypothesise that there will be lower cytotoxic effector molecule GzmB expression in

Gram-negative patient group compared to controls, based on my work in BP (Chapter 4). In order to evaluate to what extent any differences between Gram-negative sepsis and healthy controls were specific to Gram-negative sepsis, we also examined MAIT cells in a population of patients recruited from Dhaka Medical College's intensive care unit who had non-infectious critical illness such as chronic obstructive airways disease or stroke.

6.2. Aims

A. To track the phenotype, activation and function of MAIT cells in patients infected with acute Gram-negative bacteria from Bangladesh and Thailand (*E. coli*, *Salmonella* Typhi, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*).

B. To compare these responses with healthy endemic control and critically ill non-infectious patients from the same setting.

6.3. Results

6.3.1. Identification of optimum concentration of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella* Typhi.

A titration experiment of the stimulants (*K. pneumoniae*, *P. aeruginosa* and *S.* Typhi) was performed to identify the optimal concentration to stimulate IFN-γ secretion over 18 hours by MAIT cells. PF *K. pneumoniae*, *P. aeruginosa* and *S.* Typhi were used in titration and subsequent experiments. The experiment was undertaken using Oxford healthy samples. For all the organisms, different MOI (25, 50, 75) were tried. SEB

was used as a positive control. For *K. pneumoniae,* there was no difference in the number of MAIT cells between different MOIs and SEB (Figure 6.1 A). Higher IFN-γ expression was found with MOI 50 and 75 (50/75 *K. pneumoniae* per THP1 cells) although the difference did not reach statistical significance (Figure 6.1 B). We selected MOI 50 to keep it consistent with the MOI for BP used in the previous experiments. In the case of *P. aeruginosa* and *S.* Typhi, we noticed significantly higher MAIT cell frequency with MOI 75 (75 *P. aeruginosa* and *S.* Typhi per THP1 cells) compared to other MOIs (Figure 6.1 C & E). IFN-γ secretion was higher with MOI 50 compared to other MOIs in case of both *P. aeruginosa* and *S.* Typhi (Figure 6.1 D & F). Based on these findings, MOI 50 for *P. aeruginosa* and *S.* Typhi were used for all subsequent experiments. In summary, an MOI of 50 has been used in this thesis for all bacteria (BP, *K. pneumoniae*, *P. aeruginosa* and *S.* Typhi, with the exception of *E. coli* where an MOI of 30 was used, because this was optimal for *E. coli*, and historically the Klenerman group has used this MOI.

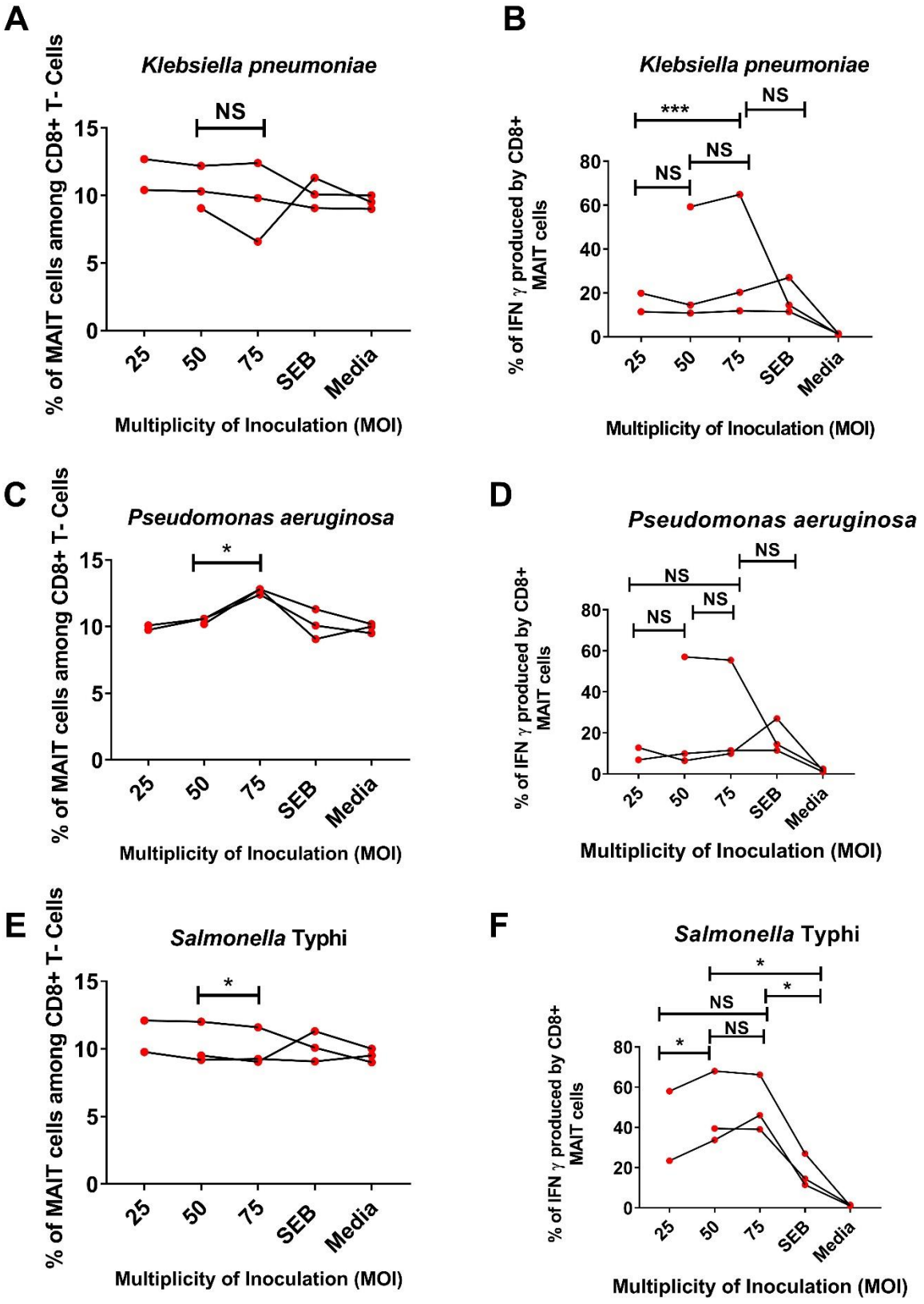

Figure 6.1. Percentage of MAIT cells and IFN-γ produced by MAIT (CD8+ T) cells after stimulation with *K. pneumoniae* (**A** and **B**), *P. aeruginosa* (**C** and **D**) and *S.* Typhi (**E** and **F)** using different MOI. Each Line and dot represent individual donors (healthy samples). Student's paired two-tailed t-test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

6.3.2. IFN-γ expression by MAIT cells is reduced in patients infected with different Gram-negative bacteria compared to healthy controls.

Adult febrile patients admitted to hospital in Bangladesh and infected with different Gram-negative organisms were studied (Chapters 2 and 3). Cases were confirmed by positive culture of blood, pus, urine or sputum. MAIT experiments were performed *ex-vivo* (without stimulation), and to elucidate the IFN-γ production, cells were stimulated overnight (18 hours ± 1 hour) with the bacterial species corresponding to the infecting organism for each patient - *K. pneumoniae*, *P. aeruginosa*, *S.* Typhi or *E. coli*. We also used the PBMC of healthy participants (from the blood donation bank) and critically ill non-infectious cases admitted to the same hospital as a control to compare the immune response. Gating was performed on the lymphocyte and T cell populations as shown in Figure 4.1.

No significant reduction in the number of MAIT cells was found (Figure 6.2 A-C) between Gram-negative infected patients (n=18) and healthy controls (n=6). However, the frequency of MAIT cells was significantly reduced in critically ill non-infectious cases (n=5) compared to healthy controls in all MAIT cell compartments (p= 0.0332). Compared to Gram-negative cases, MAIT cell frequency in critically ill non-infectious cases was also reduced in CD8+and DN MAIT cells. The ability of MAIT cells to express IFN-γ was significantly suppressed in Gram-negative bacterial patients (n=12-21) compared to healthy controls (n=22-23) in CD8+ (Figure 6.2 D, p=0.0021) MAIT cells. A reduced trend was also noted in CD8+ and DN combined MAIT cells (Figure 6.2 F). The median frequency for Gram-negative bacteria patients and healthy controls was 7.2% versus 20.0% on CD8+ MAIT cells (Figure 6.2 D) and 7.7% versus 17.0% on combined MAIT cells (Figure 6.2 F) respectively. No significant difference was found in IFN- γ expression for the DN MAIT cell population (Figure 6.2 E).

IFN- γ expression was also significantly stunted in critically ill non-infectious cases (n=11-15) compared to healthy control (n=22-23) amongst CD8+ MAIT cell compartment (p=0.0332) but not in DN or combined. No significant difference in IFN-γ expression was noted between the Gram-negative cohort and critically ill non-infectious cohort for any of the MAIT cell compartment.

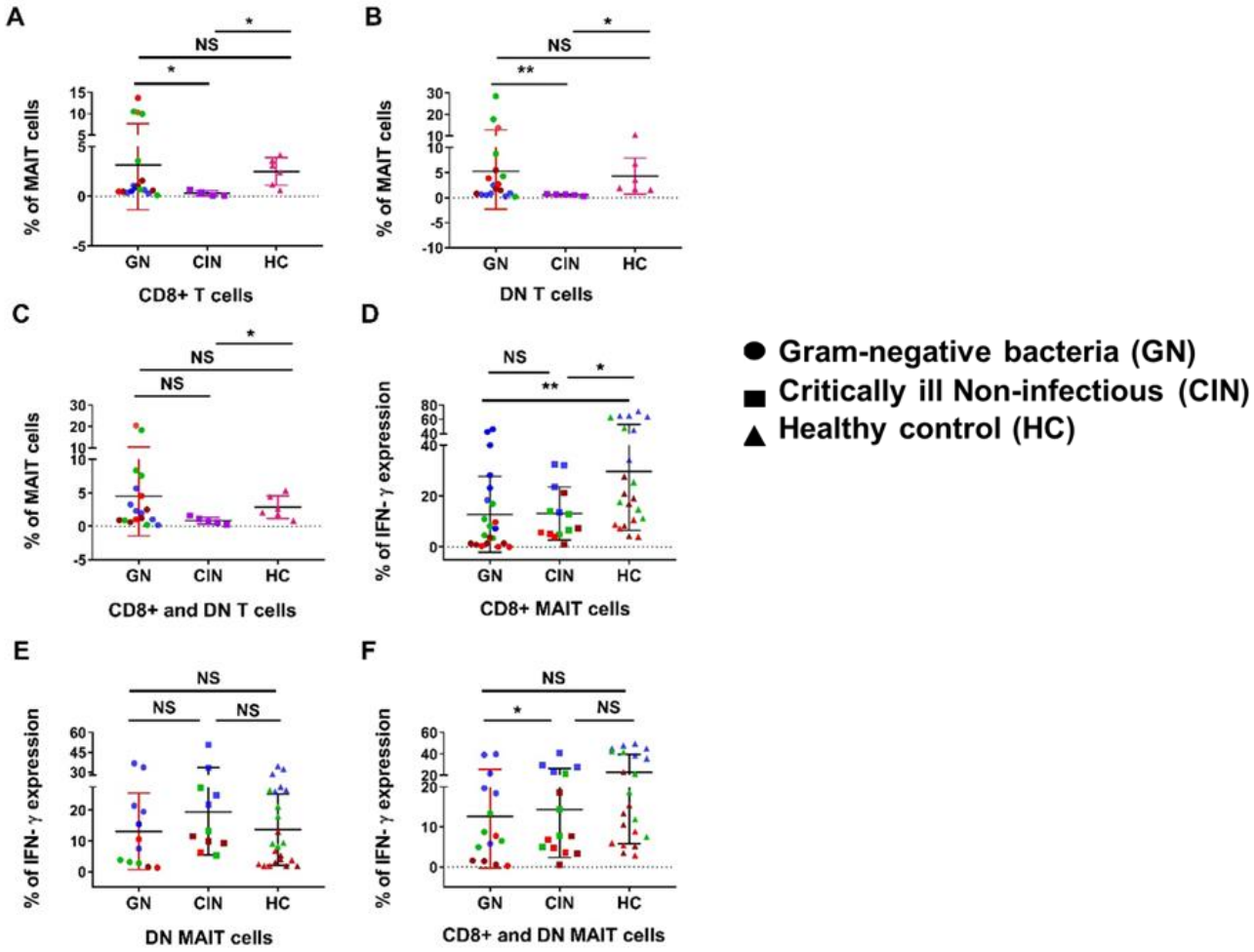

Figure 6.2. Comparison between Gram-negative bacteria infected cases, healthy controls and critically ill non-infectious cases in terms of frequency (%) of MAIT cells and IFN-γ expression. A. The frequency of MAIT cell *ex-vivo* in CD8+, **B.** DN and **C.** combined CD8+DN T cell population were compared between Gram-negative cohort (n=18), healthy control (n=6) and critically ill non-infectious cases (n=5). **D.** Percentage of IFN-γ production by CD4-CD8+ MAIT cells, **E.** DN MAIT cells, **F.** CD8+DN MAIT cells. The comparison was performed between the groups with 18 hours ± 1 hour stimulation. Every single point represents a case, and different colours represent different organisms (Maroon- *K. pneumoniae*, Red- *P. aeruginosa*, Green- *S.* Typhi and Blue- *E. coli*). For panels D, E and F, PBMC from the critically ill non-infectious controls and the healthy controls were stimulated with each of the four bacteria, and results combined on one graph. Data are presented as a scatter plot with a line at the mean ± SD. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

6.3.3. The cytotoxic effector molecule GzmB is significantly higher in Gram-negative patients compared to healthy controls, without any difference in activation.

The gating strategy to identify MAIT cells in CD8+DN T cell compartments was the same as described in Figure 4.1 and 4.6. MAIT experiments were performed *ex-vivo* (without stimulation) to elucidate the CD69 and GzmB expression. We also used the PBMC of healthy participants and critically ill non-infectious cases admitted to the same hospital as a control to compare the responses. No difference was noted for CD69 expression between Gram-negative cohort (n=17) and healthy controls (Figure 6.3 A). However, The MAIT cells were significantly activated in both the groups compared to critically ill non-infectious cases (n=5, p=0.0021). The CD69 expression in critically ill cases was almost nil.

The cytotoxic marker GzmB expression was significantly higher (median frequency 46.6% versus 6.3%; p=0.001) in the Gram-negative patient cohort (n=18) compared to healthy control (n=6). Interestingly, we found significantly higher GzmB expression amongst critically ill non-infectious cases (Figure 6.3 B) compared to the Gram-negative bacterial cohort (median percentage 88.6% versus 46.6%) and healthy control (median percentage 88.6% versus 6.3%, p=0.0021).

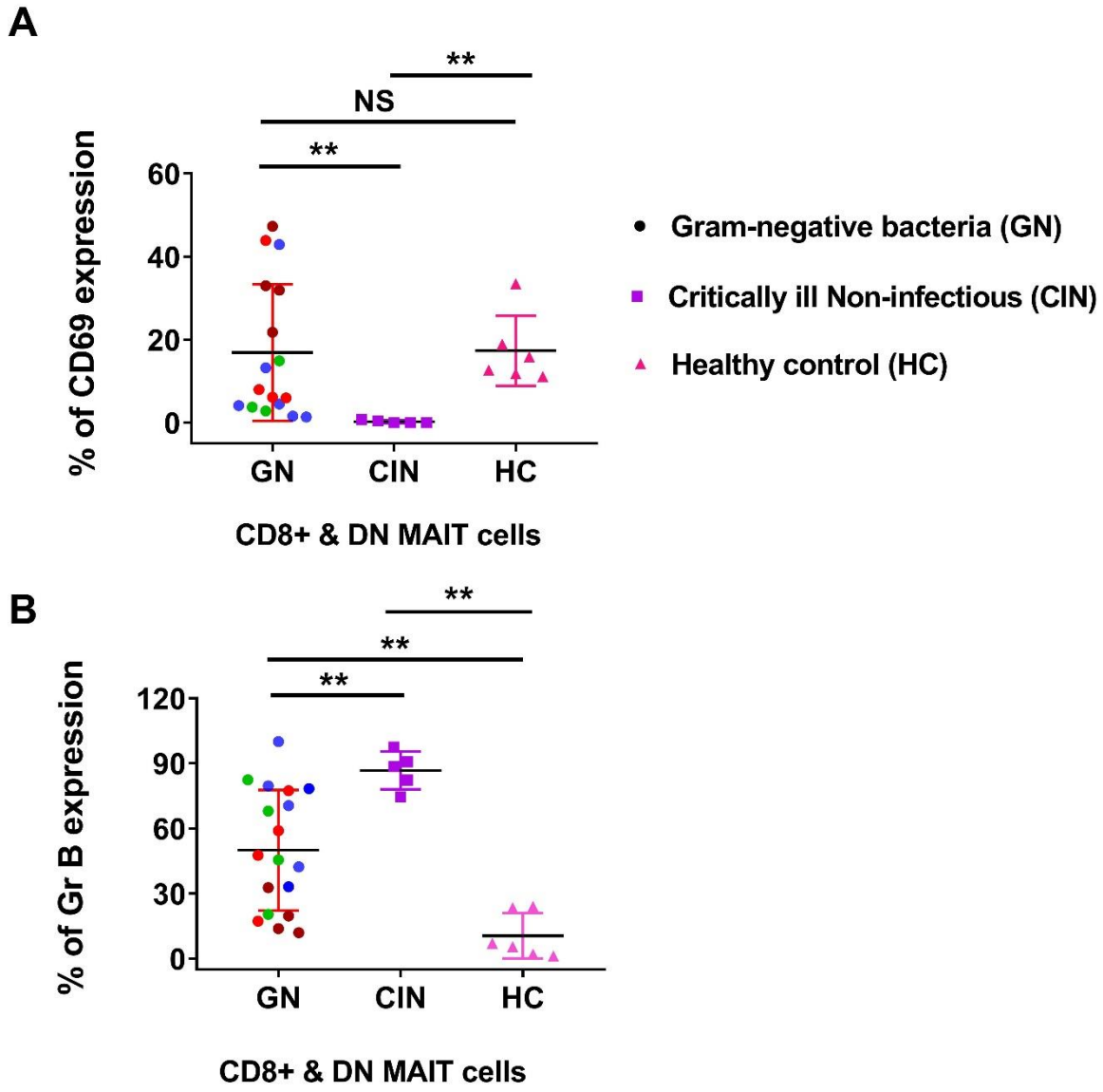

Figure 6.3. Comparison of expression of the activation marker CD69 and cytotoxic effector molecule GzmB between Gram-negative bacteria infected cases, healthy controls and critically ill non-infectious cases (*ex-vivo*). A. Percentage of CD69 expression, **B.** Percentage of GzmB expression. Every single point represents a case and different colours represent different organisms (Maroon- *K. pneumoniae*, Red- *P. aeruginosa*, Green- *S.* Typhi and Blue- *E. coli*). Data are presented as a scatter plot with a line at the mean ± SD. Mann-Whitney test was applied to identify the level of significance (****p≤0.0001; ***p≤0.001; **p≤0.01; *p≤0.05).

6.4. Discussion

We previously demonstrated evidence in Chapter 4 that the frequency of MAIT cell and IFN-γ secretion by MAIT cells are important for the survival of melioidosis patients. Having identified the potential role of MAIT cells in BP, we then set out to evaluate the role of MAIT cells in other common Gram-negative organisms responsible for sepsis, to see to what extent this was a general Gram-negative sepsis phenomenon. This is the first study to demonstrate and characterise the MAIT cell response to *K. pneumoniae*, *P. aeruginosa*, *S*. Typhi and *E. coli* infection in acutely ill febrile patients. Initially, an optimisation experiment with the organisms was carried out (except *E. coli*, which was already established in our laboratory). We found an MOI of 50 (as for BP) was the optimal concentration to use as a stimulant. For *E. coli* MOI 30 was used as for previous experiments in the Klenerman laboratory. We studied cells from acutely infected patients, and compared results with healthy controls and critically ill non-infectious cases. A previous study by another group in the U.S. reported marked reduction of MAIT cell frequency in *Streptococcus pneumonia*-infected sepsis patients compared to healthy and critically ill non-infectious controls (Grimaldi et al., 2014). We have not seen any difference in MAIT cell frequency between the Gram-negative cohort and healthy control. However, IFN-γ secretion by CD8+ MAIT cells was significantly stunted in Gram-negative sepsis patients compared to healthy controls. Our findings were partially supported by the experiments of Bourhis Le *et al.* in MR1-deficient mice, where both the percentage of MAIT cell and IFN-γ secretion were low compared to uninfected mice after different MOIs of *E. coli* infection (Le Bourhis et al., 2010).

I did not find any difference in CD69 expression between Gram-negative patients and healthy controls, although GzmB expression was significantly higher in the Gram-negative cohort. However, Bourhis Le *et al* reported higher activation (CD69 and CD25 expression) in iVα19-transgenic MAIT cells compared to controls after *E. coli*, *K. pneumoniae* and *P. aeruginosa* infection (Le Bourhis et al., 2010). Expression of cytokines IL1β, TNF-α, IL17, IL6, MIP-1α, and IL10 was found to be significantly suppressed in MR1 knock out mice compared to wild-type mice 2 days after challenge with *K. pneumoniae* (Georgel et al., 2011), which supports my results. Suppressed cytokine production was also significantly associated with severity of infection and survival. Shaler *et al* revealed prompt and robust production of the pro-inflammatory cytokines IFN-γ and TNF-α by MAIT cells (CD8+CD4-, DN and CD4+CD8-) after stimulation of human PBMC with *Klebsiella lysate* in a cytokine-dependent manner (Shaler et al., 2017). They also found higher activation (CD69) and rapid exhaustion (higher TIM-3) of MAIT cells followed by cessation of IFN-γ and TNF-α in comparison with healthy controls. The same findings were also revealed after stimulation with SEB and super antigen; potent exotoxins secreted by *Staphylococcus aureus* and *Streptococcus pyogenes* (Shaler et al., 2017). In another recent study performed on C57BL/6 mice after infecting (intra-nasal inoculation) with *S.* Typhimurium, enriched accumulation of MAIT cells in the lungs and low count in the blood was found (Chen et al., 2017). The percentage of CD8+ MAIT cells releasing the pro-inflammatory cytokines IL-17 and IFN-γ was higher in MR1 +/+ after 7 days of infection.

In *Pseudomonas*-infected cystic fibrosis patients, the number of CD8+ MAIT cells were found to be significantly lower compared to healthy controls, and lower MAIT cell numbers correlated with disease severity (Smith et al., 2014). In healthy volunteers, Kurioka *et al*. reported that, upon activation with *E. coli*, CD8+ MAIT cells

expressed significantly higher GzmB and perforin compared to controls, and had a rapid killing effect (Kurioka et al., 2015). This finding matched my result. They also revealed that this rapid upregulation is primarily MR1-dependent, with a contribution from cytokines, and is associated with Blimp1 and T-bet expression. In a recent *S.* Typhi challenge study of healthy volunteers in Oxford, CD8+ MAIT cell frequency sharply decreased after 72 and 96 hours of post-infection compared to controls (subjects without challenge) irrespective of dose (Salerno-Goncalves et al., 2017). They also reported higher activation (CD38 and HLA-DR), higher exhaustion (CD57 expression) and apoptosis (caspase-3 expression) amongst *S.* Typhi-challenged volunteers compared to controls after 48 and 96 hours. This study also found a dose-response effect, with volunteers receiving low-dose challenge having higher levels of CD38 co-expressing CCR9, CCR6, and Ki67 during infection compared to high-dose challenge (Salerno-Goncalves et al., 2017). Another challenge study with *S.* paratyphi of 25 healthy volunteers also detected lower frequency of CD8+ MAIT cells between 4 to 8 days after infection compared to controls (Howson et al., 2018). This study also found higher expression of CD38 and Ki67 in the challenged group as for in the *S.* Typhi study mentioned previously. MAIT cell frequency recovered at day 28 after antibiotic treatment (Howson et al., 2018). However, I have not found any differences in frequency of MAIT cell and activation while comparing Gram-negative patients and healthy controls.

I revealed interesting findings in the critically ill non-infectious control cohort. This cohort includes patients with acute exacerbation of COPD, acute pesticide poisoning, acute ischaemic stroke and diabetic ketoacidosis. The frequency of MAIT cells in every compartment was significantly lower in this group compared to healthy controls. The IFN-γ expression was low in CD8+ MAIT cells along with low activation and significantly higher GzmB expression compared to healthy controls. The role of

MAIT cells in these types of critical conditions have yet to be explored. A recent review predicted a potential protective role for MAIT cells in critically ill conditions like acute trauma, ischaemia re-perfusion injury after stroke and myocardial infarction, acute exacerbation of COPD, post-burn injury and others (Kim and Oldham, 2019). A study over 110 participants of childhood asthma and subsequent follow up concluded that higher MAIT cell frequency at year 1 is associated with lower risk of developing childhood asthma at age 7 (Chandra et al., 2018). CD8+ and DN MAIT cell frequency were found to be significantly lower in the blood and sputum of COPD patients compared to healthy controls (Szabo et al., 2017). Increased GzmB and perforin expression by CD8+ T cells, NK cells and macrophage have been noted in a number of studies performed in acute asthma and COPD patients compared to healthy control (Annoni et al., 2015, Hodge et al., 2006, Ngan et al., 2009). Higher GzmB level was found in the lung tissue of acute fatal asthma cases *post mortem* compared to controls (Annoni et al., 2015). Higher GzmB release by CD8+ T cells was also revealed in brain tissue of fatal acute ischaemic stroke cases compared to non-ischaemic control (Chaitanya et al., 2011). The reason behind this is possibly inflammation, rapid cell death (apoptosis) and protease-antiprotease imbalance. Further longitudinal clinical studies to examine the relationship between MAIT cells, disease severity and survival in non-infectious critical illness are warranted, alongside animal models of non-infectious critical illness to examine causality and mechanisms of effect.

6.5. Limitations

The major limitation of this chapter is the sample size. Because of the low sample size, we have compiled all the Gram-negative samples into one cohort. Therefore,

the differences in MAIT cell response to individual bacterial species could not be explored. We did not attempt to examine the relationship between MAIT cell frequency or IFN- γ release with survival in either the Gram-negative sepsis cohort or the non-infectious critical illness cohort, due to small numbers and heterogeneity within the groups. Larger longitudinal clinical studies in critical illness patients with and without infection to further characterise the role of MAIT cells are warranted. Characterising exhaustion markers may assist in explaining mechanisms of lower release of IFN-γ by MAIT cells in sepsis and non- infectious critical illness – further work is planned in our laboratory.

As for the work in previous chapters, my studies are limited by only studying the peripheral MAIT cell population, and tissue-resident MAIT cells may vary in frequency and function during acute illness. In addition, it is not possible to ascertain causality of MAIT cell changes and disease in these observational studies.

6.6. Conclusions

In conclusion, disruption of MAIT cell functionality could be an important consequence of Gram-negative sepsis, as evidenced by low IFN-γ release and higher cytotoxic molecule GzmB expression. To the best of our knowledge, this is the first experiment performed with different Gram-negative patients admitted to hospital with features of sepsis.

7. Conclusions

My interest in melioidosis started when I was working as a general medicine consultant in Bangladesh in 2013. During my clinical work, I became aware that at least two patients who died under my care had missed diagnoses of melioidosis. This was very depressing for me and I later discussed these cases with senior professors including my mentor Prof M.A. Faiz. Their opinion was that melioidosis was not a common disease, and beyond a few sporadic case reports, we lacked concrete evidence for the presence of melioidosis in Bangladesh. Prof Faiz encouraged me to investigate the presence of melioidosis in Bangladesh, and a Commonwealth PhD scholarship allowed me the opportunity to undertake this endeavour, alongside learning experimental immunology, which is a grossly under-developed research field in Bangladesh.

The aetiology of fever and sepsis has not been well studied in Bangladesh to date, and no studies have prospectively looked for melioidosis among febrile patients. Our study of the aetiology of fever in Bangladesh was a substantial undertaking because I first had to work with local microbiologists to optimise laboratory practice and recognition of BP, as well as solve many logistic and political issues, but this has paved the way for future studies in the region. I recruited 201 consecutive adults admitted with fever to either DMC or BIRDEM Hospital in Dhaka during the three-month rainy season of 2017, and found BP to be the joint commonest cause of bacteraemia along with *E. coli,* with 6/201 patients having positive cultures for each of BP and *E. coli*. Other identified organisms were *S.* Typhi, *K. pneumoniae* and *P. aeruginosa,* which are known to be common pathogens responsible for sepsis in South Asia. All six melioidosis patients had diabetes, and five out of six of them died despite prompt initiation of antibiotics (Ceftazidime or Meropenem). This frequency of BP isolation was especially surprising given the fact that the study was conducted in

Dhaka, which is an urban environment, while melioidosis is considered a rural soil-borne disease. It is likely that these patients acquired BP outside of Dhaka, and came to the city for treatment of their illness. Therefore, we believe that an even higher frequency of BP isolation from blood culture would be found if we conducted similar studies from the areas of Bangladesh that are suspected to be hotspots for melioidosis, based on case reports and evidence in soil (northern and eastern parts of Bangladesh). Around 95% of our patients received ceftriaxone (a third-generation cephalosporin antibiotic) before admission to the study sites, and this is likely to have skewed our findings towards isolation of bacteria with partial or full resistance to ceftriaxone. AMR is a growing global concern, particularly ESBL, MRSA and MDR-typhoid, and we need further studies on AMR in Bangladesh. Our study in 2017 optimised the laboratory to identify BP, and raised awareness for melioidosis, and during the same period in 2018, the laboratory diagnosed six cases of BP bacteraemia outside of a study setting, which adds validity to my study and demonstrates “real-world” impact.

The human immune defence against BP remains to be fully characterised, and further understanding of mechanisms of susceptibility to BP in diabetes and other at-risk groups is needed to guide targeted therapeutics and vaccine development. In murine models, BP is a potent inducer of IFN-γ and IFN-γ-inducing cytokines like IL-2, IL-18 and TNF-α both *in vitro* and *in vivo*. Our laboratory previously showed evidence that in human Thai acute melioidosis patients, strong CD4+ and CD8+ T cell responses are crucial for survival. In particular, the very low CD8+ T cells seen in fatal cases support the role of CD8+ T cell responses in defence against this deadly bacterium. Based on those findings I was interested to look deeper at the subsets of T cells, specifically MAIT cells. MAIT cells are a T cell subset and are abundant in the liver, lung and blood, comprising 1-10% of peripheral blood T lymphocytes. MAIT

cells are found to be activated by microbes (both Gram +/-) that possess riboflavin precursors in their synthesis pathway. BP, *S.* Typhi, *K. pneumoniae*, *E. coli*, *Pseudomonas* and other bacteria produce riboflavin during metabolism. The majority of MAIT cells are either CD8+ or double-negative (DN) T cells, although CD4+ MAIT cells are also described. This thesis suggests that MAIT cells could play an important role in survival after BP infection. In acute melioidosis, I have revealed significantly lower IFN-γ expression by MAIT cells in patients who died compared to survivors. This difference was more pronounced amongst DN and combined (CD8+ DN) MAIT cell populations. I compared the acute patients' MAIT cell response with the endemic population, and revealed a significant reduction in both in the frequency of MAIT cell (in DN compartment) and IFN-γ secretion. These data are consistent with the idea that MAIT cells play a protective role, but an alternative explanation is that the more severe the illness, the greater the negative impact on MAIT cell function.

In this thesis, I found evidence of the importance of the DN MAIT cell compartment in the blood (around 15% of total MAIT cells in the periphery) in host defence against acute BP infection. MAIT cells were found to be highly activated in acute patients compared to different controls (melioidosis recovered patients and endemic population), without any difference in GzmB expression. The activation marker CD69 was also significantly higher in acute fatal cases compared to survivors. Therefore, the reason behind this functional incapability is probably 'activation-induced cell death'. The other explanation could be 'apoptosis' as this is the hallmark of sepsis. I found IFN-γ expression upon BP stimulation is mediated by both MR1 and cytokine-dependent pathways, with a greater predominance of cytokine-dependency. Other available published work also support our findings.

Diabetes is a major risk factor for melioidosis. The burden of diabetes is increasing alarmingly in South and South East Asia where melioidosis is endemic. Therefore, I

further examined difference in MAIT cell functional, phenotype, activation and exhaustion characteristics between endemic controls and melioidosis-recovered patients with or without DM. I found no difference in the frequency of MAIT cells between diabetic and non-diabetic patients amongst the groups. In melioidosis-recovered patients (sampled at week 52 post-infection), no difference was found in IFN-γ secretion by MAIT cells amongst DM and non-DM. However, in the endemic control population IFN-γ production was significantly lower in DM compared to non-DM. I further looked at MAIT cell activation, phenotype and exhaustion markers to find out the reason for this lower IFN-γ release, but no significant difference was noted.

Gram-negative bacteria are common causes of community-acquired infection in adults in the tropics. They are also the commonest cause of sepsis in Asia. During our fever aetiology study in Bangladesh, I managed to collect PBMC from all culture-confirmed patients infected with Gram-negative bacteria (*E. coli*, *S.* Typhi, *K. pneumoniae* and *P. aeruginosa*). I examined the functional, activation and phenotypic role of MAIT cells in this cohort and compared them with healthy controls and critically ill non-infectious cases. I found no difference in the frequency of MAIT cells between Gram-negative and healthy controls. However, IFN-γ production by CD8+ MAIT cells was significantly lower in the Gram-negative patients compared to healthy controls. The cytotoxic effector molecule GzmB expression was significantly higher in Gram-negative patients compared to controls without any difference in CD69 expression. My study benefitted from an experimental approach that was individualised to each subject in the study, whereby each PBMC sample was stimulated with formalin fixed bacteria of the same species as the subject's bacteraemia (i.e. PBMC from a patient enrolled with *K. pneumoniae* bacteraemia were stimulated with fixed *K. pneumoniae*, whilst PBMC from a patient with *S.* Typhi

were stimulated with fixed *S.* Typhi). The results show the same pattern as for the MAIT cell response to BP, suggesting an important host defence mechanism against Gram-negative bacteria. To our knowledge, this is the first data to demonstrate the role of MAIT cells in humans with Gram-negative sepsis.

8. Limitations

The work in this thesis has a number of limitations as follows:

1. Because of limited resources and funding, I conducted our prospective fever aetiology study to find out the burden of melioidosis in the city of Dhaka, where I was able to build upon an existing diagnostic microbiology laboratory with an excellent microbiologist. Melioidosis is a soil-borne disease whereas Dhaka is concrete. Conducting the study in more rural regions of Bangladesh that are believed to hotspots (based on case reports, soil composition and modelling) is likely to have identified a higher frequency of melioidosis, but I have been able to demonstrate the need for microbiologists and clinicians in Bangladesh to be alert for melioidosis, even in Dhaka.
2. The group size for the MAIT cell activation, phenotype and exhaustion experiments was small, due to the limited number of PBMC samples in the archive and limited time.
3. Experiments with acute patient samples can be very challenging, and I encountered low yield of cell number retrieved from cryopreserved PBMC samples on a number of occasions. Moreover, the PBMCs were cryopreserved since 2012. We do not know how the long duration of storage and maintenance of shipment temperature could affect our findings, although

our laboratory does have some in-house data showing that method of shipping (liquid nitrogen versus dry ice) does not greatly affect the findings.

4. I could not address the question of exhaustion and apoptosis in acute melioidosis patients and endemic controls, due to the limitation of samples and time, which could further explain the lower functional response.
5. MAIT cells in the peripheral blood may not represent the body's entire MAIT cell population in terms of numbers and functionality. Published studies suggest that migration of MAIT cells to the site of infection or to the AT in case of obesity and type 2 DM may be significant. More invasive tissue sampling in human subjects in health and disease, and use of animal models are required to address this issue.
6. The biggest limitation for the Gram-negative patient cohort is the sample size. Because of the low sample size, I have compiled all the Gram-negative samples into one cohort. Therefore, the differences in individual organisms, and differences between survived and died cases, could not be explored.

9. Future Work

1. Having established laboratory capacity in diagnostic microbiology in Bangladesh, the next step for evaluating the presence of melioidosis in Bangladesh is to conduct a systematic study in hotspot areas of Bangladesh where melioidosis is predicted to be common, with integrated immunology studies.
2. It will be interesting to do future experiments with PD-1 and TIM-3 to look at exhaustion and Caspase 3 or Annexin IV for apoptosis in acute melioidosis patients.

3. Use of tetramers for specific identification of MAIT cells could allow further relevant tools to study this cell group. This approach could also help to identify MAIT cells in AT, which will further help to answer the question of migration of these cells in type 2 DM.
4. Further studies on the cellular metabolism of MAIT cells, including key metabolic factors such as glycolysis (rate, capacity and reserve), mitochondrial respiration rate, production and usage of ATP and describing the mitochondrial dynamic processes (mitochondrial dynamics, mitophagy and biogenesis) to compare amongst DM vs Non-DM, survived vs dies, acute vs endemic would be very interesting.
5. Larger longitudinal clinical studies in critically ill patients with and without infection are required to further characterise the role of MAIT cells. Characterising exhaustion and apoptosis markers may assist in explaining mechanisms of lower release of IFN-γ by MAIT cells in sepsis and non-infectious critical illness compared to healthy controls.

Concluding remarks

In this thesis, I have demonstrated that melioidosis is an important disease in Bangladesh and one of the commonest causes of hospital admission for adult febrile patients. I also explored the functional role of MAIT cells in BP and other Gram-negative bacterial infections, and found evidence that this cell subset plays a crucial role in survival and delivering a protective immune response. In addition, people with DM who are otherwise well appear to have lower MAIT cell functionality compared with non-DM healthy controls.

I will be returning to Bangladesh after completing this DPhil to take up a position of Assistant Professor with both a clinical leadership role and a research laboratory. I plan to set up integrated clinical and immunological studies to evaluate melioidosis, other Gram-negative sepsis and AMR in Dhaka and in suspected hotspots in Bangladesh. Our laboratory in Oxford will also continue work to answer further questions about the role of cellular metabolism and apoptosis in the host response to Gram-negative infection, and our collaboration will continue.

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Annex 01: Ethical permission from OXTREC for Bangladesh project

Oxford Tropical Research Ethics Committee

University of Oxford
Research Services, University Offices
Wellington Square, Oxford OX1 2JD
Tel. +44 (0) 1865 (2)82106
E-mail: oxtrec@admin.ox.ac.uk

Dr. Fazle Rabbi Chowdhury
Nuffield Department of Medicine
Peter Medawar Building for Pathogen Research, Room 305.40.19
South Parks Road
Oxford
OX1 3SY

12 January 2016

Dear Dr. Chowdhury,

Full Title of Study: Is melioidosis a significant cause of febrile admissions for people with and without diabetes in Bangladesh?

OxTREC Reference: 51-16

Thank you for your letter of 20 December 2016 in which you have responded to the Committee's request for further clarification.

I am pleased to confirm that approval has now been granted for this study. This is valid for the first five years and is subject to receiving the local ethical approval (if this approval has not yet been received).

The documents approved for this study are as follows:

Documents:	Version:	Date:
OxTREC Application Form		
Protocol	V2.0	20/12/16
PIS – patients	V1.0	12/10/16
PIS – controls	V2.0	20/12/16
ICF – patients	V1.0	12/10/16
ICF – controls	V1.0	12/10/16
Letter to volunteers with diabetes		
Peer Review Form		
PI Signature Form		

Any subsequent changes to the application must be submitted to the Committee as an Amendment. This should include a letter to give the reasons for the proposed modifications and all revised documents with changes tracked.

Tel: +44 (0)1865 (2)82106
Email: oxtrec@admin.ox.ac.uk
Web: www.admin.ox.ac.uk/rso/

Oxford Tropical Research Ethics Committee

University of Oxford
Research Services, University Offices
Wellington Square, Oxford OX1 2JD
Tel. +44 (0)1865 (2)82106
E-mail: oxtrec@admin.ox.ac.uk

Dr. Fazle Rabbi Chowdhury
Nuffield Department of Medicine
Peter Medawar Building for Pathogen Research
South Parks Road
Oxford
OX1 3SY

19 October 2017

Dear Dr. Chowdhury,

Full Title of Study: Is melioidosis a significant cause of febrile admissions for people with and without diabetes in Bangladesh?

OxTREC Reference: 51-16

Thank you for your letter of the 17 October 2017 requesting an amendment to the above study. The revised documents that you have submitted for consideration are as follows:

Documentation:	Version:	Date:
Protocol	V4.0	13/10/17
ICF – healthy controls	V2.0	12/10/17
PIS – healthy controls	V3.0	13/10/17
PIS – patients	V2.0	12/10/17

I am pleased to inform you that this amendment has been approved.

Yours sincerely

Ms Sami Kelsh
Research Ethics Administrator, OxTREC

Direct Line Tel: +44 (0)1865 (2)82106
Email: oxtrec@admin.ox.ac.uk Web: https://researchsupport.admin.ox.ac.uk/governance/ethics

Annex 02: Ethical permission from DMC for Bangladesh project

ঢাকা মেডিকেল কলেজ
DHAKA MEDICAL COLLEGE
Dhaka, Bangladesh

Ref: Memo No. MEU-DMC/ECC/2015/97(D) Date: 04/06/2017

Ethical Clearance Certificate

The Ethical Committee of Dhaka Medical College Approved the Following Research Protocol in time.

Title of the Research Work:	**"Is Meliodosis a significant cause of febrile admission for people with and without diabetes in Bangladesh ?".**
Principal Investigator:	**Dr. Fazle Rabbi Chowdhury, FCPS** Ph.D. Candidate Peter Medawar Building for Pathogen Research University of Oxford, UK.
Supervisor:	**Self**
Place of Study:	**Department of Medicine** Dhaka Medical College Hospital, Dhaka.
Duration:	**June 2017 to May 2018.**

Prof. Dr. Nasima Sultana
Head of the Department of Biochemistry &
Chairman, Ethical Review Committee
Dhaka Medical College, Dhaka.

Dhaka Medical College, Dhaka-1000, Bangladesh. Phone: 880-2-55165088, Fax : 880-2-55165006
E-mail : principal@dmc.gov.bd, dmc_principal@yahoo.com, web: http:/www.dmc.gov.bd

Annex 03: Ethical permission from BIRDEM for Bangladesh project

বাংলাদেশ ডায়াবেটিক সমিতি
DIABETIC ASSOCIATION OF BANGLADESH

Memo No. BADAS-ERC/EC/17/0127 Date: July 30, 2017

To
Dr. Fazle Rabbi Chowdhury, FCPS
PhD candidate
Peter Medawar Building for Pathogen Research
University of Oxford, UK

Subject: Ethical Clearance

The Ethical Review Committee (ERC) of the Diabetic Association of Bangladesh (BADAS) has approved your protocol on " Is Meliodosis a significant cause of febrile admission for people with and without diabetes in Bangladesh ?".

(Dr. KMS Aziz)
Chairman
Ethical Review Committee

122 KAZI NAZRUL ISLAM AVENUE SHAHBAG DHAKA 1000 BANGLADESH PHONE : 58616641-50, 880-2-9661551 FAX : 9677772, Web : www.dab-bd.org

Annex 04: Informed consent sheet of Bangladesh project

Informed Consent Form (ICF)

For Patients with Fever

Title of Study: Is melioidosis a significant cause of fever for people with and without diabetes in Bangladesh?

Date……Month……..……Year……

I _____ (name), have had the research explained to me using information sheet version _____ dated _____.

_____ *(Name of study staff whom obtained consent)* has informed me in details about:

- Objective of the study and times requires taking part in the study
- Procedures required to be performed
- Expected benefits from the research
- Potential risks of taking part in the research
- Compensations and costs for which I am responsible

I have understood all that has been read and had my questions answered satisfactorily.

***Please insert the boxes below where relevant*:**

I □ agree □ do not agree to myself taking part in this research

I □ allow □ do not allow my blood and other samples remaining from this study to be stored and used for 10 years for future research

I □ allow □ do not allow my blood to be stored and used further if I withdraw from the study

I □ allow □ do not allow my blood to be used to study genes relevant to control of infectious diseases

I □ allow □ do not allow my blood to be sent to a research institute outside Bangladesh

I understand that I / the participant can change my mind at any stage without affecting further services and hospital care to which I am entitled in the future. To consent to take part in this study I allow the investigators to use my personal information obtained from this research. My information will be presented as part of research result without revealing my / their name or identity.

Signature of participant: _____ Date

_____

Name of participant: _____ Time

_____

***OR for use if the participant lacks capacity to give consent*:**

Signature of participant's representative: _____ Date

_____

Name of participant's representative: _____ Time

_____

Only necessary if the participant cannot read or write:

I cannot read or write but the investigator / study staff have read the information in this informed consent form to me and explained it so I fully understand the given information. Therefore I provide my thumbprint to voluntary consent to taking part in this research study.

Right thumb print of the participant

Signature of witness*:_____

Name of witness*:

_____

Date: _____ Time: _____

**A witness is a person who is independent from the trial or a member of staff who was not involved in gaining the consent.*

***For the investigator / designee*:**

I certify that I have followed the study SOP to obtain consent from the participant. S/he apparently understood the nature and the purpose of the study and consents to participation in the study. S/he has been given opportunity to ask questions which have been answered satisfactorily.

Signature of investigator/designee: _____ Date

_____

Name of investigator/designee: _____ Time

_____

THE PARTICIPANT SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP

Informed Consent Form (ICF)

For Healthy/ Patient Control Volunteer

Title of Study: Is melioidosis a significant cause of fever for people with and without diabetes in Bangladesh?

OxTREC reference number: 51-60

Date……Month……Year……

I _____ (name), have had the research explained to me using information sheet version _____ dated _____.

_____ *(Name of study staff whom obtained consent)* has informed me in details about:

- Objective of the study and times requires taking part in the study
- Procedures required to be performed
- Expected benefits from the research
- Potential risks of taking part in the research
- Compensations and costs for which I am responsible

I have understood all that has been read and had my questions answered satisfactorily.

***Please insert the boxes below where relevant*:**

I □ agree □ do not agree to myself taking part in this research

I □ allow □ do not allow my blood and other samples remaining from this study to be stored and used for 10 years for future research

I □ allow □ do not allow my blood to be stored and used further if I withdraw from the study

I □ allow □ do not allow my blood to be used to study genes relevant to control of infectious diseases

I □ allow □ do not allow my blood to be sent to a research institute outside Bangladesh

I understand that I / the participant can change my mind at any stage without affecting further services and hospital care to which I am entitled in the future. To consent to take part in this study I allow the investigators to use my personal information obtained from

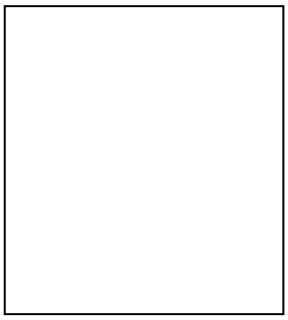
this research. My information will be presented as part of research result without revealing my / their name or identity.

Signature of participant: _____ Date _____

Name of participant: _____ Time _____

Only necessary if the participant cannot read or write:

I cannot read or write but the investigator / study staff have read the information in this informed consent form to me and explained it so I fully understand the given information. Therefore I provide my thumbprint to voluntary consent to taking part in this research study.



Right thumb print of the participant

Signature of witness*:_____

Name of witness*: _____

Date: _____ Time: _____

**A witness is a person who is independent from the trial or a member of staff who was not involved in gaining the consent.*

***For the investigator / designee*:**

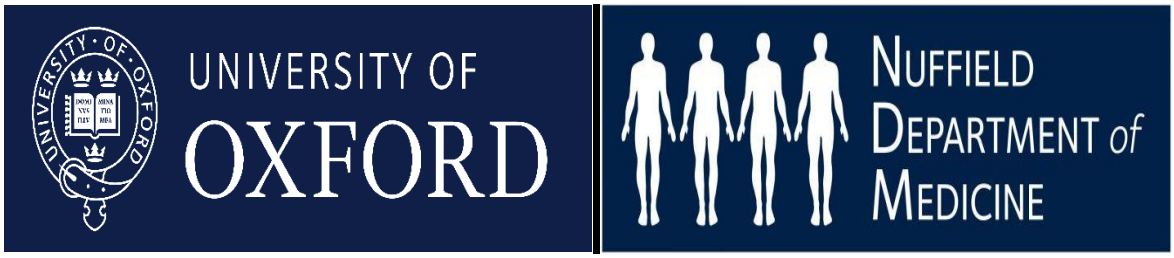
I certify that I have followed the study SOP to obtain consent from the participant. S/he apparently understood the nature and the purpose of the study and consents to participation in the study. S/he has been given opportunity to ask questions which have been answered satisfactorily.

Signature of investigator/designee: _____ Date _____

Name of investigator/designee: _____ Time _____

THE PARTICIPANT SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP

Annex 05: Patient information sheet (for patients) of Bangladesh project



Participant Information Sheet (PIS)

For Patients with Fever

Title of Study

Is melioidosis a significant cause of fever for people with and without diabetes in Bangladesh?

OxTREC reference number: 51-60

What is the study about?

Infectious diseases are still the leading cause of death in Bangladesh. When somebody comes to hospital with a fever, the doctors often do not know what disease is causing the fever. Therefore, many patients are treated with antibiotics without confirming the bug. This carries the risks of incorrect treatment and the inappropriate use of antibiotics with costs. Melioidosis is an infectious disease caused by bacteria called *Burkholderia pseudomallei*. Recently it was found that melioidosis might be a hidden cause of fever and death in Bangladesh. The variety of symptoms and lack of diagnostic facilities and manpower make this bug a major threat for Bangladesh.

In this study, we will look for the proportion of melioidosis cases among patients with fever and with or without diabetes. We can identify the other causes of fever through this study. We will also do other investigations to see how your body responds to bacteria, to help us understand why some people get ill, and to help us develop new vaccines against bacteria. Currently a large outbreak of Dengue and Chikungunya fever is undergoing. We would also like to see how our body respond to these viruses. We are recruiting 200 patients like yourself with fever, 25 healthy volunteers and 25 non-infectious acutely ill cases so that we can compare how people with illness like yourself respond to bacteria and virus with how healthy people respond to bacteria and virus.

Why have I been invited to take part?

You have been admitted to this hospital with a high fever. In this study, we want to look for the causes of your fever.

What is involved for me?

If you choose to take part in this study, you will be asked to give blood (15 ml, or approximately 3 tablespoons). Your doctors may want to also take a sample of other body fluids (e.g. pus from an abscess or fluid from an infected joint) to identify the bug. This will be part of your regular clinical care. The blood taken will be sent to the microbiology laboratory to see if it is possible to grow bacteria from the blood. Some of the blood will be used for a blood test to see if you have diabetes or not (HbA1c). If your tests come back positive for melioidosis or other Gram-negative bacteria, then we will collect an additional 15 ml (approximately 3 tablespoons) of blood from you as part of further investigation. Currently we are in the middle of Dengue and Chikungunya fever outbreak in Bangladesh. If your blood report comes out positive for Dengue or Chikungunya infection (without any bacterial infection), we will collect an additional 15 ml (approximately 3 tablespoons) of blood from you. In that case, a total 30 ml (approximately 6 tablespoons) will be collected. The collection of blood will be done by a doctor or by a trained senior nurse. We will also ask for some basic personal information from you and collect information on your illness history. You will also need to provide your contact address and telephone number to us. After 28 days, we will communicate with you by telephone to check how you are.

Expected length of time: Taking part in our study will take around 15 minutes for the first time (baseline), 10 minutes for a second blood sample if you test positive for bacteria or virus, and 5 minutes for follow up telephonic conversation. The total length of time that participants will be in the study is one month.

What will happen to my data/samples?

Your personal and disease-related information will be recorded onto a form, which will not contain your personal details that could identify you. The information will be stored in a password-protected database. Full confidentiality will be maintained for the data. Our research assistants who are doctors will be employed to collect and record your information. The information will be kept for a minimum of 3 years on paper and for a minimum of 5 years in a computer after completion of the study. Only the Principal and Co-investigators are allowed to see your information. Your blood and other samples (if taken) will be stored in a freezer for up to 10 years in Bangladesh for future research and some of them may be transferred to Oxford, UK for further study. These further studies may include tests to see how cells from your body's protection system respond to bacteria and virus, "genotyping", where we compare parts of your

genetic make-up with how you respond to infection, and if you have diabetes, tests to find out what kind of diabetes you have.

Your personal information will not be disclosed to others. When the study is completed, we will combine your test results with those of the other participants, and the overall results will be analysed. We may wish to share these overall results with the scientific community, for example by publishing a scientific paper, in which case we will make sure, that you cannot be personally identified. Only the overall observations of a group of anonymous patients will be reported. A further application to the ethics committees will be made if we wish to perform new studies not outlined in the study protocol. At any stage, you can instruct us to destroy stored samples by contacting Dr. Lovely Barai (Co-investigator of this study), Associate professor and Head of Microbiology, BIRDEM hospital, Dhaka, Tel. No: +0088- 01711364510.

Are there any risks in taking part?

There is no significant physical or psychological risks of participating in this study. The collection of samples might cause minimum pain, local bleeding and bruising which usually do not requires any treatment, but the clinical team if required would provide appropriate medical care.

What are the benefits of taking part?

You will receive your printed blood culture report (test to look for the bug) free of charge. In addition a test to identify diabetes (HbA1C) will be performed free of charge too and a paper copy of the report will be given to you.

Do I have to take part?

No. Taking part in research is voluntary. You are also free to change your mind at any point during the study without giving any reason. If you withdraw from the study, the blood sample collected before your withdrawal would be used unless you specifically request otherwise. If you do not agree to the study, you will be treated the same as other patients in the hospital; it will not negatively affect your treatment. You may ask questions at any time.

Has the study been reviewed by an ethics committee?

The ethical committee of the hospital where you are admitted (DMCH/ BIRDEM) has reviewed and approved the study. You can contact the ethical committee if needed. The address for Dhaka Medical College Hospital is The Office of the Principal, Dhaka Medical College, secretariat road, Dhaka. Bangladesh, Tel: +880 1911-032927 and for BIRDEM is, The Office of the Director Hospital, 122,Kazi Nazrul Islam Avenue,Shahbagh,Dhaka 1000, Bangladesh, Tel: +880 2-8616644.

What if I have any questions or want to raise a concern?

If you have any questions or concerns after reading this leaflet, you will have a chance to discuss them fully with a member of our team before you decide whether to take part. We will also be available throughout the study to answer any questions or address any concerns that you may have later on.

Names of the doctors I can contact in case of any problems or question.

The principal investigator: Dr. Fazle Rabbi Chowdhury, address: Tropical Disease Research Cell, Dhaka Medical College building- 02 (fifth floor), Dhaka-1205, Cell no- 01916578699.

Co-investigator (for DMC): Dr. Md. Robed Amin, Associate professor, Department of Medicine, cell no- 01711725787.

Co-investigator (for BIRDEM): Dr. Kaniz Fatema, Assistant professor, Department of Critical Care Medicine, cell- 01715038849.

If you want to ask someone independent anything about this research, please contact:

Dr. Khan Abul Kalam Azad, Professor and Head, Department of Medicine, Room No- 512 (building -02). Dhaka Medical College, Dhaka, Bangladesh. Tel: +0088- 01818282152.

Annex 06: Case Record Form of Bangladesh project

DEMOGRAPHICS
Sex: O Female O Male **Age**: I__I__I__I years **Date of birth**: |D|D| |M|M| |Y|Y|
Ethnicity: O Bangladesh O Other ____________ **Hospital Reg. No:**
Residential address with Upazilla and District and mobile number:
Height: I__I__I__I cm O N/A **Weight**: I__I__I__I kg O N/A
Occupation:______________________ ***Distance from home to hospital***: I__I__I__I km
Current exposure to rice field *(within last 6 months)* YES / NO O N/A
Current exposure to other crops field (within last 6 months) YES / NO O N/A
Religion: Muslim O Hindu O Cristian O Buddhism O Tribal O Others O

BEFORE THIS ADMISSION
If Known Diabetes, Treatment for Diabetes (recent 3 months before this admission) O N/A

Drug (generic name)	**Duration of Treatment** (choose one)	**Route** (choose one)
O Metformin O Glipizide O Gliclazide O Glimepiride O Glibenclamide O Pioglitazone O Acarbose O Minidiab O Insulin O Other____________________________________	O ≤ 2 weeks O > 2 weeks – 3 months O > 3months O N/A	O po O sc O im O iv O other O N/A
O Metformin O Glipizide O Gliclazide O Glimepiride O Glibenclamide O Pioglitazone O Acarbose O Minidiab O Insulin O Other____________________________________	O ≤ 2 weeks O > 2 weeks – 3 months O > 3months O N/A	O po O sc O im O iv O other O N/A
O Metformin O Glipizide O Gliclazide O Glimepiride O Glibenclamide O Pioglitazone O Acarbose O Minidiab O Insulin O Other____________________________________	O ≤ 2 weeks O > 2 weeks – 3 months O > 3months O N/A	O po O sc O im O iv O other O N/A
O Metformin O Glipizide O Gliclazide O Glimepiride O Glibenclamide O Pioglitazone O Acarbose O Minidiab O Insulin O Other____________________________________	O ≤ 2 weeks O > 2 weeks – 3 months O > 3months O N/A	O po O sc O im O iv O other O N/A
O Metformin O Glipizide O Gliclazide O Glimepiride O Glibenclamide O Pioglitazone O Acarbose O Minidiab O Insulin O Other____________________________________	O ≤ 2 weeks O > 2 weeks – 3 months O > 3months O N/A	O po O sc O im O iv O other O N/A

ON ADMISSION

Admission date: | D | D | | M | M | | Y | Y | **First presentation**: O ICU O IPD O Emergency

Name of the admission department:

Admission Diagnoses: 1) _____ 2) _____ 3) _____

Main symptom leading to hospitalization (tick all apply)

O fever O cough O confusion O GI Symptoms O Headache O Rash

O Bleeding O Neck Stiffness O urinary symptoms O joint pain O malaise

O Unconscious O Skin/ subcutaneous abscess O other (specify) _____

Duration of symptom before hospitalization: | I I I | days

AT ENROLMENT ***Date*** | D | D | | M | M | | Y | Y | ***Time***: | | | : | | | (24:00)

Screen date: | D | D | | M | M | | Y | Y | ***Time***: | | | : | | | (24:00)

Inclusion Criteria (please circle)

Age ≥ 18 years YES / NO

History of fever >38 degrees in first 48 hours of admission YES / NO

Willingness to participate in the study and written, informed consent obtained from the patient or their representative YES / NO

Vital signs during admission:

Body temperature: | I I I . | | ^{o}F Heart rate: | I I I | / min

Blood pressure: | I I I | / | I I I | mmHg Respiratory rate: | I I | / min

Pulse Oximeter level (**If available)**: | I I I | %

***Oxygen support at the time*:** YES / NO O N/A

If YES: O O_2 rate | I I | l/min O FiO_2 | I I I | % O Air O Unknown

If YES: O Cannula O Mask O Mask e Bag O Electrical Ventilator O Others (specify) _____

Blood Results (recent results from admission to enrolment, which are available) ***Date*** | D | D | | M | M | | Y | Y |

O N/A

Hb | | | • | | g/dL Hct | | | • | | % WBC | | | , | I I I | cells/mm^3 Neutrophil | | | % Lymphocyte | | | % Monocyte | | | % Basophil | | | % Eosinophil | | | % Platelet | | , | I I I | , | I I I | cells/mm^3 ESR | I I I | CRP I I I I BUN | I I I | mg/dL S. Cr | | | • | | mg/dL AST | | | | | iU/L ALT

|__|__|__| iU/L ALP |__|__|__| iU/L Lactate |__|__|__| mEq/L

RBS |__|__|__| mg/dL Total Bilirubin |__|__|•|__| mg/dL Albumin |__|__|•|__|

g/dL HBA1C |__|__|•|__| %

HBsAg: Positive/Negative. Value: HBc Ab Total:

CXR:

USG:

Other tests (If done):

Core investigations check list (Please tick while collected):

Date and time of collection	Blood sample (in volume)			Other Type of Sample	Tests done						**If BC positive for Gram negative**		
	10 ml for culture	3 ml in EDTA for HbA1C	3ml serum for IHA	1. 2. 3.	Blood culture	Other culture	Serology/ IHA	HBA1C	PCR	Others	15 ml for PBMC	3 ml serum	3 ml plasma

Isolates collected and stored (for Burkholderia only): YES / NO ○ N/A **Storage Code:**

Plasma (ml) collected and stored: YES / NO ○ N/A **Storage Code**:

Serum (ml) collected and stored: YES / NO ○ N/A **Storage Code**:

PBMC (ml) collected and stored: YES / NO ○ N/A **Storage Code:**

Blood Culture report:

Other Culture report:

HbA1C value: **IHA (will be done later):** ○ Positive ○ Negative

Other available reports:

Culture positive site (tick all positive where applicable)

○ Blood ○ Urine ○ Sputum ○ Throat swab ○ Pus ○ Others: ……

Organ involvement if Melioidosis (tick all those that apply) ○ Septic shock (BP<90mmHg systolic) ○ Lung ○ Liver ○ Spleen ○ Skin ○ Prostate ○ Renal/urinary ○ Throat ○ Blood ○ Bone/Joint ○ Other (specify) _____ ○ Neurological ○ Deep seated abscess

Risk factors/ Co-morbidities (please circle)

Diabetes (diagnosed by doctor) YES / NO

If YES: 1) Insulin dependent YES / NO 2) Oral drug treatment YES / NO

Renal Disease (eCrCl < 60ml/min/ Creatinine level > 1.5 mg) YES / NO Creatinine level: ……..

If YES: 1) Peritoneal dialysis YES / NO 2) Hemodialysis YES / NO

Chronic Lung Disease YES / NO

If YES: 1) Asthma YES / NO 2) COPD YES / NO

Chronic Liver Disease YES / NO

If YES: 1) Hepatitis YES / NO Type of Virus:

Chronic Heart Disease YES / NO

If YES: 1) Coronary heart dis YES / NO 2) Congestive failure YES / NO

Stroke/vascular Disease YES / NO **Steroid intake** (<8 wks) Yes/ NO

Malignancy/Cancer YES / NO

If YES: 1) Chemo YES / NO 2) Congestive failure YES / NO

Previous Tuberculosis (≥ 6 months before this enrolment) YES / NO

If YES: Date /estimate last previous culture or diagnosis if known **Date** | D | D | | M | M | | Y | Y |

Hypertension YES / NO

Other Diseases YES / NO If YES: specify _____

Current alcohol excess (>2 drinks/day) YES / NO **Smoking** YES / NO

Previous melioid / typhoid/ any other infectious disease YES / NO If YES: What was the diagnosis:

Date /estimate last previous culture or diagnosis if known **Date** | D | D | | M | M | | Y | Y |

Name of the Medications and duration during hospital stay (from admission to discharge, only core drugs)

DISCHARGE STATUS ***Date of discharge or death***: | D | D | | M | M | | Y | Y |

○ **Diagnosis at discharge:**

○ Completed iv Rx & started oral Rx ○ Died in hospital ○ Discharged but died at home

○ Not improved and discharged on risk bond ○ Transferred to other hospital

Medications during Discharge (only mention the core drugs)

Drug (generic name)	**Dose/Times**	**Route** (choose one)
1. _____	Dose (mg) | | | | | ○ N/A___ Times per day | | | ○ N/A	○ po ○ sc ○ im ○ iv ○ other ○ N/A
2. _____	Dose (mg) | | | | | ○ N/A___ Times per day | | | ○ N/A	○ po ○ sc ○ im ○ iv ○ other ○ N/A

3. _____ __	Dose (mg) | _ _ _ _ | O N/A___ Times per day | _ _ _ | O N/A	O po O sc O im O iv O other O N/A
4. _____ __	Dose (mg) | _ _ _ _ | O N/A___ Times per day | _ _ _ | O N/A	O po O sc O im O iv O other O N/A
5. _____ __	Dose (mg) | _ _ _ _ | O N/A___ Times per day | _ _ _ | O N/A	O po O sc O im O iv O other O N/A

FOLLOW-UP DAY 28

Telephonic conversation YES / NO ***Date*** | D | D | | M | M | | Y | Y |

If No (specify): O Moved from region O Unable to contact O Died (Date | D | D | M | M | Y | Y |) O Unknown O Other

Current status

O Remains Unwell O Well O Died O Unknown O Drug defaulter

If unwell any new symptoms since hospital discharge YES / NO If YES (tick all apply)

O fever O muscle aches O fatigue O body weakness O weight loss O skin rash

O jaundice O headache O cough O shortness of breath O vomiting O diarrhoea

O poor appetite O pain with urination O joint swelling O chest pain

O other pain, site:_____ O bleeding, site:_____

O weakness of body part: _____ O numbness of body part: _____

O swelling of body part: _____ O other, describe: _____

What medications are you taking now (including antibiotics) O N/A

Drug (generic name)	Drug (generic name)
1.	3.
2.	4.
5.	6.
7.	8.

Follow-up appointment made in unwell cases: YES / NO If NO: state reason _____

Form completed by: _____Signature: _____ Date: | D | D | | M | M | | Y | Y |

Counter Signature of the PI/ Co-Investigator: _____ Date: | D | D | | M | M | | Y | Y |

Annex 07: Letter to the control participants diagnosed as DM

Date:

To

…….

……

……

Dear ……..

Thank you very much for taking part in this study as a volunteer. We did a blood test called 'HbA1C' to diagnose Diabetes Mellitus with your blood.

Your blood test came back POSITIVE for Diabetes.

Diabetes is a common, treatable disease which is caused by high sugar in the blood. If you do not control your high sugar, you are at increased risk of health problems like heart attack, stroke, kidney disease and vision problems. Some people can control their blood sugar by changes in their diet and through exercise. If it is not controlled by diet and lifestyle modification, it can be controlled by a variety of medications.

Firstly you need to re-confirm your diagnosis. For that purpose, I would like to advise you to contact the Medicine out-patient department (OPD) of Dhaka Medical College hospital, Ramna, Dhaka, Tel: 8626812-26 with the enclosed report. It is open on Thursday to Saturday from 8 am to 1 pm. They will evaluate you further and give appropriate advice on management.

If you have any other query, please contact us. Our contact details are Dr. Fazle Rabbi Chowdhury, address: Tropical Disease Research Cell, Dhaka Medical College building- 02 (5th floor), Dhaka-1205, Cell no- 01916578699 or Dr. Md. Robed Amin, Associate professor, Department of Medicine, cell no- 01711725787.

With thanks

Dr.Fazle Rabbi Chowdhury
Tropical Disease Research Cell
Dhaka Medical College building- 02 (5th floor),
Dhaka-1205, Cell no- 01916578699.

Annex 08: SOP for PBMC isolation (core steps)

1.0 Reagents and Materials

Item	Catalog No.	Company
L-Glutamine	G7513	Sigma Aldrich
RPMI-1640 Medium with Sodium bicarbonate, no L-Glutamine	R0883	Sigma Aldrich
Penicillin/Streptomycin	P0781	Sigma Aldrich
Fetal Calf Serum	F7524	Sigma Aldrich
Leucosep tubes	227290	Greiner Bio-one
Lymphoprep	07861	StemCell Technologies

Solutions to be made up

R0

- 500 ml RPMI 1640 (without glutamine)
- 5 ml 10000 U/ml penicillin/streptomycin from aliquot.
- 5 ml 200 mM L-Glutamine from aliquot.
- Label “R0” and write date, initials, and write PS and LG lot numbers on the bottle. Store at 4°C and replace after 1 month or if any concern about sterility

R10

- 500ml RPMI 1640 (without glutamine)
- 5 ml 10000 U/ml penicillin/streptomycin from aliquot
- 5ml 200 mM L-Glutamine from aliquot
- Add 50ml filtered heat inactivated FCS. Keep a note of the FCS lot number.
- Label “R10” and write date, initials, FCS, PS and LG lot numbers on the bottle. Store at 4C and replace after 1 month or if any concern about sterility

Samples

Whole blood from study participants collected in heparinised tubes.

2. Procedure

This method uses a filter (lymphoprep reagent and filter disc) to separate out the PBMC by density gradient centrifugation.

- Have R0 and R10 reagents (above) in the waterbath at 37°C
- Check there is 1 aliquot (2.5ml) of FCS in fridge

- Collect heparinized blood. Process the blood within 3 h. (For 6-30ml of blood you will need 1 x 50ml leucosep tube per subject, see VOLUME A. If the volume is <6ml you will need 1 x 12ml leucosep tube per subject, see VOLUME B)
- Label Leucosep tubes.

VOLUME A (10-30ml blood, use 50ml Leucosep tubes):

- Pipette 15ml Lymphoprep into each 50ml Leucosep tube. Centrifugate at approximately 1000 x g for 2 min to get the Lymphoprep below the porous filter disc.
- Pour or pipette up to 30 ml heparinized blood into each labelled Leucosep tube and centrifuge at 1000 x g for 15 min at RT without brake.

VOLUME B (<6ml blood, use 12ml Leucosep tubes):

- Pipette 4ml Lymphoprep into each 12ml Leucosep tube. Centrifugate at approximately 1000 x g for 2 min to get the Lymphoprep below the porous filter disc.
- Pour or pipette up to 6ml heparinized blood into each labelled Leucosep tube and centrifuge at 1000 x g for 15 min at RT without brake.

For both blood volume sizes then continue as follows:

- For plasma samples: Take 5 x 150µl and 1x 1-ml samples of the plasma fraction (top) and place in 1.8-ml cryovials labelled with the subject study ID and the date. Store at -80°C.
- Collect the PBMC from the white/milky interface with a pasteur pipette (take all), and place the PBMC from each leucosep tube into a new, labelled 15ml tube (50-ml falcon tube if >20ml). It is better to take some plasma and lymphoprep as well as it will get washed out. If you used multiple leucosep tubes for the same donor then you can combine the PBMC in the same tube at this point.
- Top the tubes up to 15ml (40 ml if >20ml blood) with R0, and spin at 1800 rpm 5 min RT.
- Quickly pour off the supernatant and resuspend the pellet by running the tube a few times along an eppendorf tube rack.
- Add 15-40ml of R0, spin at 1400 rpm, 7 min, RT.
- Pour off the supernatant and resuspend the pellet (as above), in EXACTLY 10 ml R10 for counting.
- Leave to rest for 1 hour approx. in 37°C CO2 humidified incubator if possible before Counting

Refer to SOP-OX-IM006 for Counting and SOP-OX-IM007 for Freezing/Thawing procedures.

Annex 09: SOP for cryopreservation and thawing of cell (core steps)

1.0 Reagents and Materials

Item	Catalog No.	Company
Fetal Calf Serum	F7524	Sigma Aldrich
DMSO	D2650-100ML	Sigma Aldrich
Isopropanol, >99.5%	10215390	Acros Organics (via Fisher Scientific)
Freezing Container, Nalgene Mr. Frosty	CRY8400	Scientific Laboratory Supplies
L-Glutamine	G7513	Sigma Aldrich
RPMI-1640 Medium with Sodium bicarbonate, no L-Glutamine	R0883	Sigma Aldrich
Penicillin/Streptomycin	P0781	Sigma Aldrich
Benzonase® Nuclease, Purity > 99% 1ku	70664-3	Merck Millipore

Solutions to be made up

R0

- 500 ml RPMI 1640 (without glutamine)
- 5 ml 10000 U/ml penicillin/streptomycin from aliquot.
- 5 ml 200 mM L-Glutamine from aliquot.
- Label “R0” and write date, initials, and write PS and LG lot numbers on the bottle. Store at 4°C and replace after 1 month or if any concern about sterility

R10

- 500ml RPMI 1640 (without glutamine)
- 5 ml 10000 U/ml penicillin/streptomycin from aliquot
- 5ml 200 mM L-Glutamine from aliquot
- Add 50ml filtered heat inactivated FCS. Keep a note of the FCS lot number.
- Label “R10” and write date, initials, FCS, PS and LG lot numbers on the bottle. Store at 4C and replace after 1 month or if any concern about sterility

Cryosolution (2x)

20% DMSO in FCS, e.g. V=10ml: add 2ml DMSO to 8ml FCS

Prepare fresh, store at 4C until use, 30min before use put on ice

Samples

Cells, e.g. PBMC

2. Procedure

FREEZING

- Make sure the Mr. Frosty is filled with Isopropanol up to the indicated line and that the Isopropanol has been replaced after 5 freeze/thaw cycles
- Make sure the Mr. Frosty is brought to 4C prior to use (min. 30min)
- Make sure the Cryprotectant (2x) is ice-cold (place in a box with ice and into the BL2 cabinet)
- Spin cells at 1800rpm 5 min
- Resuspend in FCS from the fridge:
    - 0.5 ml of FCS per vial to be frozen, always have at least 5 million cells per vial and preferably below 10 million per ml
    - I.e. if you have 20 million PBMC left to freeze, these should be frozen in 2-4 vials so that 5-10 million PBMC will be in each vial. IF you decide to freeze at 5 million per ml in 4 vials, then at this stage you will need to add 0.5 ml per vial = 0.5x4 = 2ml of FCS to the cell suspension in the 50ml falcon tube.
- Place the cells on ice for 30 min or at 4 degrees for 20mins -1hour.
- Add an equal volume of ice-cold 20% DMSO in FCS (ie 2 ml for 4 vials) to the cells to achieve a final amount of 10% DMSO in FCS v/v and QUICKLY mix by pipetting up and down.
- Aliquot the cell suspensions to cryovials (1 ml per vial) labelled with the donor ID, date and number of cells, and your initials and immediately place them in the Mr Frosty containers.
- As soon as one container is full, place it in the –80°C freezer.
- After an overnight (or possibly over weekend) incubation in the –80°C, transfer the frozen cells to liquid nitrogen if available.

THAWING

- Make sure the waterbath is on +37C
- Prepare a 15ml tube per sample to be thawed, label with the sample name in the BL2 cabinet. You can thaw up to two vials of the same donor per 15ml tube.
- Warm R0 and R10 to +37°C in the water bath.
- Add 10ml of R10 to a 15ml tube.
- Two vials of cells should be thawed at a time in your hand.
- When nearly thawed (there should still be a little bit of ice left– when you can invert the tube and the lump of ice falls it is ready) add 1ml pre-warmed R10 solution to each vial and mix by inverting tube or pipetting up and down with a Pasteur pipette.

- Transfer the mix to the 15ml tube containing 10ml R10 slowly and dropwise. Transfer some of the R10 back into the vial to wash it out. If thawing more than one vial from the same subject at a time, the mix from the 2nd vial from the same subject can be added to the same 15ml falcon.
- Spin at 1400rpm, 7min
- Discard supernatant (pour off), break pellet
- Wash with 5ml R0
- Spin at 1400rpm, 5min
- Discard supernatant (pour off), break pellet
- *Optional Benzonase treatment: if clumping of PBMC has been observed. Add 1ul of Benzonase (25U/ul, 10KU, Novagen, 70746-3) per 5ml of R10 to the PBMC suspension (final concentration 5U/ml) after the second washing step. Incubate at least 30 min at 37°C (in CO2 incubator). Pellet cells at 1400 rpm for 5 min at RT by centrifugation
- Benzonase treatment should always be performed for patient samples (e.g. Melioidosis cohort), it is optional for samples from healthy individuals. If healthy samples and patient samples are used for the same assay the healthy samples not getting Benzonase treatment still have to be incubated at the same time the patient samples are incubated in order to keep the conditions the same.
- Resuspend pellet in an appropriate volume of R10 depending on expected concentration
- Count cells as described in SOP-OX-IM006 and resuspend in the correct volume for your assay with R10.
- Keep in a 15ml tube with cap slighlty unscrewed for resting at 37C in the incubator (resting time depends on the downstream assay)

Annex 10: SOP for E. coli preparation (culture, fixation and counting)

1.0 Reagents and Materials

Item	Catalog No.	Company
LB Broth, L3022-1 kg	101983727	Sigma Life science
LB Broth with Agar (Lennox), L7025-500 tab	10024146 56	Sigma Life science
Glycerol, 500ml	CHE2070	Scientific Lab suppliers
Paraformaldehyde solution 4% (1 litre)	281692	Chem Cruz
Falcon tubes 5ml	734-0442	VWR
Falcon tubes 50ml	352070	VWR
48 well plate flat bottom	353078	Corning Science
Nalgene Polycarbonate Baffled Culture Flasks (500 ml)	4110-0500	Thermo Fisher Scientific

Solutions to be made up

For Liquid Broth

- Take 10-gram LB powder in a glass bottle.
- Pour with 500 ml miliQ water
- Label it 'LB for growth' and autoclave.
- After autoclave keep in room temperature.

For Agar plate

- Take 500 ml miliQ water in a glass bottle.
- Put 10 LB Agar tablet (1tab/50 ml).
- Label it 'LB for agar plate' and autoclave.
- After autoclave, use 10ml/ Petri dish. Use while hot and clear.
- Wait for 30-60 minutes to cool down and set. Keep them in fridge for future use.

E. coli Samples

DH5-alpha genotype collected from Klenerman's lab.

2. Procedure

Day 01: Setting of colonies in agar plate

- Take a plate, bring *E. coli* stock from liquid nitrogen, and thaw on ice.
- Put the loop into vial and twirl around.
- Streak across the petri dish three times and put into incubator (designated for bacterial growth, opposite of Klenerman's lab)

Day 02: Growth of *E. coli* (work inside hood)

- Check the previous day autoclaved LB Broth is clear.
- Pour 100 ml Broth into the conical flask (previously autoclaved).
- Take out the E coli petri dish from incubator and put into fridge 30 minutes before collecting colonies.
- Take 10 colonies (1 colony/10 ml) using a 200 ul tip and put it into the conical flask. Wrap the opening with foil. Do not completely seal. Keep it loose.
- Use Parafilm tape to seal the Petri dish (containing colonies) and keep in fridge for future use.
- Take a 50 ml falcon tube, pour with 40 ml Broth and insert a 200-ul tip. This tube is for sterility control.
- Put the conical flask and falcon tube into Shaker. Use setting RPM 180-220, 37 ^{0} C. Leave over night to grow. Tube with *E. coli* should look cloudy.

Day 03: Setting and Fixation of *E. coli* (work inside hood)

- Take a 48 well plate. Use the first row. Mark them according to dilution as 1/10, 1/100, 1/1000, 10^4, 10^5, 10^6, 10^7 and 10^8.
- Bring the conical flask and falcon tube from the shaker inside hood. Take two 50 ml falcon tube and pour 40 ml in to each. Keep the rest 20 ml for storage purpose.
- Fill the first row with 450 ul sterile and filtered (use round filter to prepare them) PBS. Take 50 ul *E. coli* from the flask and add into first well.
- Mix several times and take 50 ul to add in next well. Mix several times. Do the same for successive wells to get the desired dilution.
- Spin down the falcon tubes (including sterility control) @ 4000 rpm for 8 minutes.
- Remove the supernatant, re-suspend and wash the pallet once in 10 ml of PBS.
- Take out six agar plates from the fridge and level them as 10^4, 10^5, 10^6, $10^{7,}$ 10^8 and sterility control.
- Put 3-4 beads (reusable from Klenerman lab) in each dish and put 50 ul from the corresponding well into petri dish. Use 50 ul sterile PBS for sterility control.

- Manually move all the petri dishes together 8-10 times in each direction (4 directions). The idea is to smoothly spread the bacteria into petri dishes.
- Do not discard the bead; soak them into virkon and autoclave for re-use.
- Keep the petri dishes into designated incubator for 12-16 hours maximum. If it is more than that, the counting of colonies will be difficult as they started to merge and thick.
- Thaw 4% PFA in water bath. Take two-falcon tube and pour with 15 ml in each tube. Add 15 ml sterile PBS in each tube to make the composition 2% PFA.
- Add the 30 ml 2% PFA in to previously wash *E. coli*. First, put 2-3 ml PFA into tube, mix them with 1000ul pipette, then add the rests.
- Leave at RT for 20 minutes.
- Spin down @ 4000 rpm for 7-8 minutes, remove supernatant, re-suspend and wash with 30 ml sterile PBS for once/ twice.
- Re-suspend in **3 ml** filtered sterile PBS (kept on ice). Use 1000ul pipette for accurate measurement instead of 5 ml stripette.
- Keep them in fridge for next day aliquot after counting.

Storage of E. coli (do it on ice):

- Take 10 one ml cryo vial and level them as DH5-ά.
- Put 500 ul of *E. coli*.
- Add 500 ul Glycerol. Use filtered pipette, as glycerol is very thick and sticky.
- Mix them several times and put into -80 freezer.

Day 04: Counting and preserve

- Take the 10^6 plate for counting colonies. Also, check 10^5 and 10^7 and SC plate. Count the number of colonies (CFU's) carefully. There should be no colonies in SC plate.
- Multiply the number of colonies (CFU/50 ul) with dilution factor.
- The count will be in million/6000 ul (as total 6ml in two tubes).
- Convert them into million/ul.
- Take 500ul in each Eppendorf and store them in fridge for use or -20 freezer for long-term storage.

Annex 11: SOP for *B. pseudomallei* preparation (culture, fixation and counting)

Whole cell formalin fixed *B.psudomallei* (Bp) & *B.thailandensis* (Bt)

Bacterial subculture from frozen stock

1. Leave a vial of frozen bacteria in room temperature to thaw
2. Use 10ul loop to inoculate bacteria from frozen stock onto TSB broth and incubate overnight
3. Transfer bacteria from overnight culture onto Ashdown agar using 1ul loop, incubate 24-48 hours

Formalin fixation

1. Use 10ul loop to pick bacterial colonies and suspend in 20ml PBS
2. Measure optical density (OD) of the mixture using spectrophotometer at 600nm, then adjust OD to be approx. 1.0 (5x10^8 cells/ml, add more PBS if needed
3. Perform colony count to calculate CFU/ml for cell suspension (spread 50ul from each dilution of cell suspension on agar plate and count colonies, e.g., 10^5, 10^6, 10^7)
4. Transfer 20ml cell suspension to a new 50ml tube and spin at 3500 rpm for 20 minutes, discard supernatant
5. Add 1.0 ml 0.125% (0.04 M) formaldehyde and transfer entire cell suspension to 2.0ml tube
6. Incubate at 37^{o}C overnight
7. Wash 3 times by spinning the mixture at 8000rpm for 5 minutes in PBS
8. Re-suspend cell pellet in 1.0ml PBS
9. Confirm bacterial killing by;
    a. Plating 50ul cell suspension onto Columbia agar and 50ul onto TSB broth, incubate at 37^{o}C for 48 hours and observe Bp growth
    b. Plating 50ul of TSB (a) onto Columbia agar, incubate at 37^{o}C for 48 hours and observe Bp growth
    c. If there is no growth of Bp detected, thus bacterial killing is confirmed

Notes: % formaldehyde can be varied which depends on bacterial viability.

Sonication

1. Sonicate cell suspension five times on ice for 20 seconds with 1 minute interval
2. This fraction will contain bacterial membrane and intracellular components for further experiment

Annex 12: Protocol for Flow cytometry staining: extracellular (ECS) and intracellular (ICS) for MAIT cells functional response (core steps)

1.0 Reagents and Materials

Item	Catalog No.	Company	Comment
96 well round bottom plates non-sterile, PS	735-0180	VWR	Use for regular sized samples (>1x105c)
96 well V bottom plates non-sterile, PS	735-0184	VWR	Use for small sample size (<1x105c)
Falcon tubes 5ml, no cap	734-0442	VWR	Use if only a few samples are stained, and for compensation
Compensation beads (mouse IgG kappa)	552843	BD Biosciences	Use to setup compensation on BD LSRII
ArC™ Amine Reactive Compensation Bead Kit (for use with LIVE/DEAD™ Fixable dead cell stain kits)	A10346	Thermo Fisher (Life Technologies)	This is used for compensation staining of the blue live/dead stain
Live/Dead Fixable Cell Stain Near Infrared (633nm, APC-H7 channel) Live/Dead Fixable Violet Dead Cell Stain Kit (Pacific Blue channel)	L10119 L34955	Thermo Fisher (Life Technologies)	To distinguish live from dead cells on fixed samples
Phosphate buffered saline tablets	P4417-100TAB	Sigma Aldrich	
Sodium Azide	S2002-5G	Sigma Aldrich	Stabilizer
BSA	A2153-50G	Sigma Aldrich	Protein carrier
Formaldehyde solution (Formalin) with 10-15% methanol as stabilizer	S2BF0690V	Sigma-Aldrich	For fixation and permeabilization
eBioscience Permeabilization buffer	00-8333-56	eBioscience	For extracellular and

			intracellular cytokine/chemokine/growth factor staining
eBioscience FOXP3/Transcription Factor Staining Buffer Set	00-5523-00	eBioscience	For intracellular/ intranuclear transcription factor staining
IC Fixation Buffer	00-8222-49	eBioscience	Fixation of cells for short term storage (3 days, 4C)
FACS Lysing Solution	349202	BD Biosciences	For Red blood cell lysis of whole blood samples
Antibodies with different conjugates	various	BD, Milteny, Bio legend, eBioscience, others	Titrate before use in experiment and note titration result in Inventory

Solutions to be made up

PBS

- Made with PBS tablets from SIGMA, and dH2O. 1 tablet in 100ml water.
- Autoclave and store at RT and keep sterile.

10% Sodium Azide solution

- This substance is highly toxic! Use double gloves, DO NOT GET IN CONTACT WITH METALS (no metal spatulas) when working with the powder, do not pour down the sink at high concentration (dilute with water first)- risk of spontaneous combustion when in contact with metals. DO NOT HEAT TO 275°C. Work only under a functioning, inspected fume hood. If in contact with water it forms toxic gases!!!
- Weigh 1g Sodium Azide using a plastic spatula into a 15 or 50 ml falcon tube under the fume hood
- Add 10ml dH2O, close the bottle, let dissolve
- Store in a secondary container protected from light at 4°C (keep for 1 month)
- For a final conc. of 0.01% Sodium Azide: make a 1:1000 dilution

FACS Buffer (0.1% BSA, 0.01% Sodium Azide in PBS), alternatively you can use MACS running buffer if no FACS buffer available or plain 1xPBS (but only if acquiring within a day)

- 500 ml PBS
- 0.5g BSA
- Dissolve BSA in PBS on a magnetic stirrer
- Add 0.5ml Sodium Azide 10%
- Filter and keep at +4C
- Use within 2 weeks of preparation or discard earlier if contamination is suspected

Live/Dead Cell stain (prepare acc. to manufacturer's instructions, example for Near-IR Stain below)

- Thaw one vial of live/dead cell stain and the DMSO vial provided in the kit
- Quick spin the stain to make sure everything is in the bottom of the vial
- Re-suspend the stain in 50ul DMSO (supplied with Kit)
- Keep in the dark, use within 1 hour of preparation, aliquot rest (3ul per 0.5ml vial) and store at -80C for future use
- Use at a 1:1000 working dilution in FACS buffer or PBS

Antibody Panels

- Antibody panels have to be prepared on ice on the same day of use or one day in advance. If you want to use the same antibody panel for one experiment spanning over 1 week you can prepare the panel for all samples and use them within that week. But be aware that some conjugates are less stable than others.
- Tandem conjugates which are often used for panels are less stable than single conjugates. Tandem conjugates can break resulting in a single conjugate which could give false positive results, e.g. CD19 PE-Cy5 and CD4 PE are in the same panel: Cy5 breaks off PE-Cy5 which results in a CD19 PE antibody conjugate showing up in the same channel as CD4 PE.

2% Formaldehyde solution

- Prepare it on 37 wt % in distilled water. So dilute 2 ml formaldehyde solution with 35 ml distilled water. Store this at 4C for future use.

eBioscience FOXP3/Transcription Factor Staining Buffer Set

- Fixation/Permeabilization concentrate (Part# 00-5123) 4x.
    - 1:4 dilution: Add 1 part Fixation/Permeabilization concentrate to 3 parts fixation permeabilization diluent (Part#00-5223)
    - Always prepare fresh
- Permeabilization buffer (Part#00-8333): Dilute 10x permeabilization buffer in DI water to make a 1x solution (1:10 dilution)
    - Store at 4C, use within 1 week
- Use Kit components within 6 months of opening

Samples

PBMC or sorted white blood cells, whole blood either fresh or thawed (refer to SOP-OX-IM007). If cells have been fixed prior to freezing, they can be subjected to ECS but antibodies have to be tested for compatibility. Compare a healthy fixed control to a healthy unfixed control. Same goes for ICS staining in general, some surface antibodies might be affected by the fixation procedure, therefore their performance has to be tested before starting an experiment.

2. Procedure

Always keep cells and reagents on ice during staining and in the fridge during incubations unless otherwise indicated! Work in the dark due to photosensitivity of fluorochromes!

Setup appropriate controls for your stainings: single stained compensation controls using beads and FMOs using cells (you only have to setup FMOs once for one experiment that you are running within a few weeks, setup up fresh FMOs if the last experiment has been a while ago).

A. Live-dead Staining of mononuclear cells

- Cells are stained in 5ml Falcon tubes or 96 well plates V-bottom in case of low cell numbers (<1x10^5/well) and U-bottom in case of whole blood staining and high cell numbers (>1x10^5/well).
- Add Brefeldin A four (4) hours prior to staining.
- Centrifuge at 1800 rpm for 3 min
- Take off supernatant. Cells should form a pallet at the bottom of the wells.
- Gently tap the plate against the bench top to break the pallet.
- Add 50ul live/dead solution to each well.
- Incubate at 4^0c for 10 minutes.

B. Extra-cellular and Intra-cellular staining:

1. Add 150 ul PBS in each well.
2. Centrifuge at 1800 rpm for 3 min
3. Take off supernatant. Cells should form a pallet at the bottom of the wells.
4. Gently tap the plate against the bench top to break the pallet.
5. Add 150 ul 2% formaldehyde to each well.
6. Incubate at room temperature for 15 minutes.
7. Centrifuge at 1800 rpm for 3 min. Take off supernatant. Cells should form a pallet at the bottom of the wells. Gently tap the plate against the bench top to break the pallet.

8. Add 150 ul eBioscience permealization buffer at working concentration (see page 04) into each well.

9. Centrifuge at 1800 rpm for 3 min. Take off supernatant. Cells should form a pallet at the bottom of the wells. Gently tap the plate against the bench top to break the pallet.

10. Add 50 ul antibody cocktail into per well.

11. Incubate at room temperature for 30 minutes.

12. Add 150 ul PBS per well, centrifuge at 1800 rpm for 3 min. Take off supernatant. Gently tap the plate against the bench top to break the pallet.

13. Add 100 ul PBS/ FACS buffer per well and the plate is ready to be run on the MACSQuant.

14. Re-suspend in 300ul FACS buffer/ PBS for acquisition on LSRII.

14. Keep at 4^0c, if not run in the same day after wrapping. It can be kept for maximum 3 days.

<u>Staining of BD Compensation Beads (only possible with IgG kappa antibodies produced in mouse)</u>

- Add one drop of beads and one drop of negative control to 5ml Falcon tubes (approx. 120ul) or a 96well round bottom plate
- Add 80ul FACS buffer
- Add antibody to the concentration that you use in your sample (single stainings, per conjugate used you have to setup 1 staining, each color is stained in a separate tube/well)
- Incubate 20min, 4°C, dark
- Spin at 2000rpm, 3min
- Decant supernatant and vortex pellet
- Wash with FACS buffer (1ml for tubes, 150ul for plates), centrifuge as above
- Resuspend in 300ul FACS buffer for acquisition on LSRII or 100ul FACS buffer for MACSQuant
- Store at 4°C in the dark until acquisition, acquire the same day!

<u>Staining of Arc Compensation Beads (only for Fixable Live/Dead stain compensation)</u>

- Add one drop of Arc beads to a 5ml Falcon tube or a 96well round bottom plate
- Add 1ul freshly prepared or thawed fixable Live/Dead stain
- Incubate 20min, 4°C, dark
- Spin at 2000rpm, 3min
- Decant supernatant and vortex pellet
- Wash with FACS buffer (1ml for tubes, 150ul for plates), centrifuge as above
- Resuspend in 300ul FACS buffer for acquisition on LSRII or 100ul FACS buffer for MACSQuant
- Add 1 drop of negative control prior to acquisition
- Store at 4°C in the dark until acquisition, acquire the same day!

COMMENTS

- A maximum of 1x10^6 cells can be stained per 100ul antibody mixture. Adjust accordingly if the cell number is higher. In case of lower cell numbers: 1x10^5 cells and below use 50ul.

Annex 13: Protocol for Flow cytometry staining: extracellular (ECS) and intracellular (ICS) for MAIT cells activation, phenotype and exhaustion response (core steps)

1.0 Reagents and Materials

Item	Catalog No.	Company	Comment
96 well round bottom plates non-sterile, PS	735-0180	VWR	Use for regular sized samples (>1x105c)
96 well V bottom plates non-sterile, PS	735-0184	VWR	Use for small sample size (<1x105c)
Falcon tubes 5ml, no cap	734-0442	VWR	Use if only a few samples are stained, and for compensation
FcBlocking reagent human	130-059-901	Miltenyi Biotec	Blocking of unspecific binding (important if there are a lot of monocytes, macrophages, DC)
Compensation beads (mouse IgG kappa)	552843	BD Biosciences	Use to setup compensation on BD LSRII
ArC™ Amine Reactive Compensation Bead Kit (for use with LIVE/DEAD™ Fixable dead cell stain kits)	A10346	Thermo Fisher (Life Technologies)	This is used for compensation staining of the blue live/dead stain
Live/Dead Fixable Cell Stain Near Infrared (633nm, APC-H7 channel) Live/Dead Fixable Violet Dead Cell Stain Kit (Pacific Blue channel)	L10119 L34955	Thermo Fisher (Life Technologies)	To distinguish live from dead cells on fixed samples
Phosphate buffered saline tablets	P4417-100TAB	Sigma Aldrich	

Sodium Azide	S2002-5G	Sigma Aldrich	Stabilizer
BSA	A2153-50G	Sigma Aldrich	Protein carrier
BD Fixation/Permeabilization Kit	554714	BD Biosciences	For intracellular cytokine/chemokine/growth factor staining
eBioscience FOXP3/Transcription Factor Staining Buffer Set	00-5523-00	eBioscience	For intracellular/ intranuclear transcription factor staining
IC Fixation Buffer	00-8222-49	eBioscience	Fixation of cells for short term storage (3 days, 4C)
FACS Lysing Solution	349202	BD Biosciences	For Red blood cell lysis of whole blood samples
Antibodies with different conjugates	various	BD, Biolegend, eBioscience, others	Titrate before use in experiment and note titration result in Inventory

Solutions to be made up

PBS

- Made with PBS tablets from SIGMA, and dH2O. 1 tablet in 100ml water.
- Autoclave and store at RT and keep sterile.

10% Sodium Azide solution

- This substance is highly toxic! Use double gloves, DO NOT GET IN CONTACT WITH METALS (no metal spatulas) when working with the powder, do not pour down the sink at high concentration (dilute with water first)- risk of spontaneous combustion when in contact with metals. DO NOT HEAT TO 275°C. Work only under a functioning, inspected fume hood. If in contact with water it forms toxic gases!!!
- Weigh 1g Sodium Azide using a plastic spatula into a 15 or 50 ml falcon tube under the fume hood
- Add 10ml dH2O, close the bottle, let dissolve
- Store in a secondary container protected from light at 4°C (keep for 1 month)
- For a final conc. of 0.01% Sodium Azide: make a 1:1000 dilution

FACS Buffer (0.1% BSA, 0.01% Sodium Azide in PBS), alternatively you can use MACS running buffer if no FACS buffer available or plain 1xPBS (but only if acquiring within a day)

- 500 ml PBS
- 0.5g BSA
- Dissolve BSA in PBS on a magnetic stirrer
- Add 0.5ml Sodium Azide 10%
- Filter and keep at +4C
- Use within 2 weeks of preparation or discard earlier if contamination is suspected

Live/Dead Cell stain (prepare acc. to manufacturer's instructions, example for Near-IR Stain below)

- Thaw one vial of live/dead cell stain and the DMSO vial provided in the kit
- Quick spin the stain to make sure everything is in the bottom of the vial
- Resuspend the stain in 50ul DMSO (supplied with Kit)
- Keep in the dark, use within 1 hour of preparation, aliquot rest (3ul per 0.5ml vial) and store at -80C for future use
- Use at a 1:1000 working dilution in FACS buffer or PBS

Antibody Panels

- Antibody panels have to be prepared on ice on the same day of use or one day in advance. If you want to use the same antibody panel for one experiment spanning over 1 week you can prepare the panel for all samples and use them within that week. But be aware that some conjugates are less stable than others.
- Tandem conjugates which are often used for panels are less stable than single conjugates. Tandem conjugates can break resulting in a single conjugate which could give false positive results, e.g. CD19 PE-Cy5 and CD4 PE are in the same panel: Cy5 breaks off PE-Cy5 which results in a CD19 PE antibody conjugate showing up in the same channel as CD4 PE.

Lysing Solution (10x)

- Dilute 1 part 10x FACS Lysing Solution with 9 parts ROOM TEMPERATURE sterile WATER (not PBS). May store this at RT for future use.

BD Fixation/Permeabilization Kit

- BD Cytofix/Cytoperm is supplied as 1x (use as is)
- BD Perm/Wash: Dilute 10x BD Perm/Wash buffer in dH2O to make a 1x solution (1:10 dilution)
    - store at 4C, use within 1 week
- Use Kit components within 6 months of opening

eBioscience FOXP3/Transcription Factor Staining Buffer Set

- Fixation/Permeabilization concentrate (Part# 00-5123) 4x.

    - 1:4 dilution: Add 1 part Fixation/Permeabilization concentrate to 3 parts fixation permeabilization diluent (Part#00-5223)
    - Always prepare fresh
- Permeabilization buffer (Part#00-8333): Dilute 10x permeabilization buffer in DI water to make a 1x solution (1:10 dilution)
    - Store at 4C, use within 1 week
- Use Kit components within 6 months of opening

Samples

PBMC or sorted white blood cells, whole blood either fresh or thawed (refer to SOP-OX-IM007). If cells have been fixed prior to freezing, they can be subjected to ECS but antibodies have to be tested for compatibility. Compare a healthy fixed control to a healthy unfixed control. Same goes for ICS staining in general, some surface antibodies might be affected by the fixation procedure, therefore their performance has to be tested before starting an experiment.

2. Procedure

Always keep cells and reagents on ice during staining and in the fridge during incubations unless otherwise indicated! Work in the dark due to photosensitivity of fluorochromes!

Setup appropriate controls for your stainings: single stained compensation controls using beads and FMOs using cells (you only have to setup FMOs once for one experiment that you are running within a few weeks, setup up fresh FMOs if the last experiment has been a while ago).

A. ECS: Extracellular Staining of mononuclear cells

- Cells are stained in 5ml Falcon tubes or 96 well plates V-bottom in case of low cell numbers (<1x10^5/well) and U-bottom in case of whole blood staining and high cell numbers (>1x10^5/well).
- If cells are resuspended in FACS buffer already continue with step 7
- Centrifuge at 1800 rpm for 3 min
- Take off/decant supernatant into waste bucket
- Break pellet by vortexing
- Resuspend cells in FACS buffer
- Centrifuge at 1800 rpm for 3 min
- Take off/decant supernatant into waste bucket
- Break pellet by vortexing
- Prepare your Ab mixture in FACS buffer containing FcR blocking reagent (1:10 diluted) and live/dead cell fixable dye (if applicable)

  Note: FcR blocking using the Miltenyi product can be performed with Ab staining simultaneously. Alternatively, you can block with 100ul blocking reagent in FACS buffer for 10min at 4C, centrifuge at 1800 rpm for 3 min, flick off supernatant into waste bucket, break pellet by vortexing and then continue with Ab staining.

- Add 100ul of above Ab mixture to each well and incubate for 20min at 4C in the dark
- Centrifuge at 1800 rpm for 3 min
- Take off/decant supernatant into waste bucket

- Break pellet by vortexing
- Wash with 1ml FACS buffer/tube or 150ul FACS buffer/well and centrifuge as above
- Centrifuge at 1800 rpm for 3 min
- Take off/decant supernatant into waste bucket
- Break pellet by vortexing
- Wash with 1ml FACS buffer/tube or 150ul FACS buffer/well
- Centrifuge at 1800 rpm for 3 min
- Take off/decant supernatant into waste bucket
- Break pellet by vortexing
- Continue with **C. ICS: Intracellular staining**

or

- Fix by resuspending in 100µl FACS buffer and 100ul IC Fixation Buffer, gently resuspend by pipetting up and down. A total final volume of 200ul is necessary when acquiring on the LSRII cytometer, however when using the MACSQuant only resuspend in 50ul FACS buffer and 50ul IC Fixation Buffer.
- Store wrapped in aluminium foil at 4°C in the dark (up to 3 days, but ideally measure the next day) until acquisition

B. <u>ICS: Intracellular staining of mononuclear cells</u>

FIXATION/PERMEABILIZATION

- Add 100 µL of Fix/Perm solution per well or 250ul per tube, incubate for 20 min at 4°C in the dark
- Centrifuge the plate/tubes at 2,000 rpm for 3 min at 4°C
- Take off supernatant
- Break pellets by vortexing plate
- Add 150ul of Perm/Wash per well or 500ul per tube and let sit on ice for 5min in the dark
- Centrifuge the plate/tubes at 2,000 rpm for 3 min at 4°C
- Take off supernatant
- Break pellets by vortexing plate
- Take off/decant supernatant into waste bucket

Note: use the Fix/Perm and Perm/Wash from BD for intracellular Cytokine and Caspase 3 staining and the Fix/Perm and Perm/Wash from eBiosciences for transcription factor and Granzyme staining.

INTRACELLULAR STAINING

- Add 50µL Ab and FcR blocking reagent diluted in the appropriate Perm/Wash buffer for intracellular staining
- Incubate for 20 min at 4°C in the dark
- Centrifuge the plate/tubes at 2,000 rpm for 3 min at 4°C
- Take off supernatant
- Break pellets by vortexing plate

- Wash cells with 150 μL/well or 450ul/tube of Perm/Wash
- Centrifuge the plate/tubes at 2,000 rpm for 3 min at 4°C
- Take off supernatant
- Break pellets by vortexing plate
- Resuspend in 100ul (MACSQuant) or 200 ul (LSRII) of FACS buffer or 1xPBS
- Wrap plates in aluminum foil and store at 4°C until acquiring samples on a flow cytometer (same or next day ideally, but up to 3 days post staining).

Staining of BD Compensation Beads (only possible with IgG kappa antibodies produced in mouse)

- Add one drop of beads and one drop of negative control to 5ml Falcon tubes (approx. 120ul) or a 96well round bottom plate
- Add 80ul FACS buffer
- Add antibody to the concentration that you use in your sample (single stainings, per conjugate used you have to setup 1 staining, each color is stained in a separate tube/well)
- Incubate 20min, 4°C, dark
- Spin at 2000rpm, 3min
- Decant supernatant and vortex pellet
- Wash with FACS buffer (1ml for tubes, 150ul for plates), centrifuge as above
- Resuspend in 300ul FACS buffer for acquisition on LSRII or 100ul FACS buffer for MACSQuant
- Store at 4°C in the dark until acquisition, acquire the same day!

Staining of Arc Compensation Beads (only for Fixable Live/Dead stain compensation)

- Add one drop of Arc beads to a 5ml Falcon tube or a 96well round bottom plate
- Add 1ul freshly prepared or thawed fixable Live/Dead stain
- Incubate 20min, 4°C, dark
- Spin at 2000rpm, 3min
- Decant supernatant and vortex pellet
- Wash with FACS buffer (1ml for tubes, 150ul for plates), centrifuge as above
- Resuspend in 300ul FACS buffer for acquisition on LSRII or 100ul FACS buffer for MACSQuant
- Add 1 drop of negative control prior to acquisition
- Store at 4°C in the dark until acquisition, acquire the same day!

COMMENTS

- A maximum of 1x10^6 cells can be stained per 100ul antibody mixture. Adjust accordingly if the cell number is higher. In case of lower cell numbers: 1x10^5 cells and below use 50ul

Annex 14: SOP for *S.* Typhi preparation (culture, fixation and counting)

1.0 Reagents and Materials

Item	**Catalog No.**	**Company**
LB Broth, L3022-1 kg	101983727	Sigma Life science
LB Broth with Agar (Lennox), L7025-500 tab	1002414656	Sigma Life science
Glycerol, 500ml	CHE2070	Scientific Lab suppliers
Paraformaldehyde solution 4% (1 litre)	281692	Chem Cruz
Falcon tubes 5ml	734-0442	VWR
Falcon tubes 50ml	352070	VWR
48 well plate flat bottom	353078	Corning Science
Nalgene Polycarbonate Baffled Culture Flasks (500 ml)	4110-0500	Thermo Fisher Scientific

Solutions to be made up **(solutions and plates will be made in CAT II laboratory)**

For Liquid Broth

- Take 10-gram LB powder in a glass bottle.
- Pour with 500 ml miliQ water
- Label it 'LB for growth' and autoclave.
- After autoclave keep in room temperature.

For Agar plate

- Take 500 ml miliQ water in a glass bottle.
- Put 10 LB Agar tablet (1tab/50 ml).
- Label it 'LB for agar plate' and autoclave.
- After autoclave, use 10ml/ Petri dish. Use while hot and clear.
- Wait for 30-60 minutes to cool down and set. Keep them in fridge for future use.

S. typhi Samples

CT18 strain collected from OU NHS microbiology laboratory.

2. Procedure

Day 01: Setting of colonies in agar plate

- Take a plate, bring *S. typhi/ S. para-typhi* stock from liquid nitrogen, and thaw on ice.
- Put the loop into vial and twirl around.
- Streak across the petri dish three times and put into incubator (designated for bacterial growth, opposite of Klenerman's lab)

Day 02: Growth of *S. typhi/ S. para-typhi* (work inside hood)

- Check the previous day autoclaved LB Broth is clear.
- Pour 100 ml Broth into the plastic conical flask (previously autoclaved).
- Take out the *S. typhi/ S. para-typhi* petri dish from incubator and put into fridge 30 minutes before collecting colonies.
- Take 10 colonies (1 colony/10 ml) using a 200 ul tip and put it into the conical flask. Wrap the opening with foil and tape it tight as it is an aerobic organism.
- Use Parafilm tape to seal the Petri dish (containing colonies) and keep in fridge for future use.
- Take a 50 ml falcon tube, pour with 40 ml Broth and insert a 200-ul tip. This tube is for sterility control.
- Put the conical flask and falcon tube into Shaker. Use setting RPM 180-220, 37 ^{0} C. Leave over night to grow. Tube with *S. typhi/ S. para-typhi* should look cloudy.

Day 03: Setting and Fixation of *S. typhi/ S. para-typhi* (work inside hood)

- Take a 48 well plate. Use the first row. Mark them according to dilution as 1/10, 1/100, 1/1000, 10^4, 10^5, 10^6, 10^7 and 10^8.
- Bring the conical flask and falcon tube from the shaker inside hood. Take two 50 ml falcon tube and pour 40 ml in to each. Keep the rest 20 ml for storage purpose.
- Fill the first row with 450 ul sterile and filtered (use round filter to prepare them) PBS. Take 50 ul *S. typhi/ S. para-typhi* from the flask and add into first well.
- Mix several times and take 50 ul to add in next well. Mix several times. Do the same for successive wells to get the desired dilution.
- Spin down the falcon tubes (including sterility control) @ 4000 rpm for 8 minutes.
- Remove the supernatant, re-suspend and wash the pallet once in 10 ml of PBS.
- Take out six agar plates from the fridge and level them as 10^4, 10^5, 10^6, $10^{7,}$ 10^8 and sterility control.

- Put 3-4 beads (reusable from Klenerman lab) in each dish and put 50 ul from the corresponding well into petri dish. Use 50 ul sterile PBS for sterility control.
- Manually move all the petri dishes together 8-10 times in each direction (4 directions). The idea is to smoothly spread the bacteria into petri dishes.
- Do not discard the bead; soak them into virkon and autoclave for re-use.
- Keep the petri dishes into designated incubator for 12-16 hours maximum. If it is more than that, the counting of colonies will be difficult as they started to merge and thick.
- Thaw 4% PFA in water bath. Take two-falcon tube and pour with 15 ml in each tube. Add 15 ml sterile PBS in each tube to make the composition 2% PFA.
- Add the 30 ml 2% PFA in to previously wash *S. typhi/ S. para-typhi.* First, put 2-3 ml PFA into tube, mix them with 1000ul pipette, then add the rests.
- Leave at RT for 20 minutes.
- Spin down @ 4000 rpm for 7-8 minutes, remove supernatant, re-suspend and wash with 30 ml sterile PBS for once/ twice.
- Re-suspend in **3 ml** filtered sterile PBS (kept on ice). Use 1000ul pipette for accurate measurement instead of 5 ml stripette.
- Keep them in fridge for next day aliquot after counting.

Storage of S. typhi/ S. para-typhi (do it on ice):

- Take 10 one ml cryo vial and level them.
- Put 500 ul of *S. typhi/ S. para-typhi.*
- Add 500 ul Glycerol. Use filtered pipette, as glycerol is very thick and sticky.
- Mix them several times and put into -80 freezer.

Day 04: Counting and preserve

- Take the 10^6 plate for counting colonies. Also, check 10^5 and 10^7 and SC plate. Count the number of colonies (CFU's) carefully. There should be no colonies in SC plate.
- Multiply the number of colonies (CFU/50 ul) with dilution factor.
- The count will be in million/6000 ul (as total 6ml in two tubes).
- Convert them into million/ul.
- Take 500ul in each Eppendorf and store them in fridge for use or -20 freezer for long-term storage.

Annex 15: SOP for *K. pneumoniae* preparation (culture, fixation and counting)

1.0 Reagents and Materials

Item	**Catalog No.**	**Company**
LB Broth, L3022-1 kg	101983727	Sigma Life science
LB Broth with Agar (Lennox), L7025-500 tab	1002414656	Sigma Life science
Glycerol, 500ml	CHE2070	Scientific Lab suppliers
Paraformaldehyde solution 4% (1 litre)	281692	Chem Cruz
Falcon tubes 5ml	734-0442	VWR
Falcon tubes 50ml	352070	VWR
48 well plate flat bottom	353078	Corning Science
Nalgene Polycarbonate Baffled Culture Flasks (500 ml)	4110-0500	Thermo Fisher Scientific

Solutions to be made up **(solutions and plates will be made in CAT II laboratory)**

For Liquid Broth

- Take 10-gram LB powder in a glass bottle.
- Pour with 500 ml miliQ water
- Label it 'LB for growth' and autoclave.
- After autoclave keep in room temperature.

For Agar plate

- Take 500 ml miliQ water in a glass bottle.
- Put 10 LB Agar tablet (1tab/50 ml).
- Label it 'LB for agar plate' and autoclave.
- After autoclave, use 10ml/ Petri dish. Use while hot and clear.
- Wait for 30-60 minutes to cool down and set. Keep them in fridge for future use.

KP Samples

ATCC 13368 strain collected from OU NHS microbiology laboratory.

2. Procedure

Day 01: Setting of colonies in agar plate

- Bring *Klebsiella pneumoniae* stock from liquid nitrogen and thaw on ice.
- Put the loop into vial and twirl around.
- Streak across the petri dish three times and put into incubator (designated for bacterial growth)

Day 02: Growth of *Klebsiella pneumoniae* (work inside hood)

- Pour 100 ml Broth into the conical flask (autoclaved).
- Take out the *Klebsiella pneumoniae* petri dish from incubator and put into fridge 30 mins before collecting colonies.
- Take 10 colonies (1 colony/10 ml) using a 200 ul tip and put it into the conical flask. Wrap the mouth with foil tightly (it is facultative anaerobes).
- Take a 50 ml falcon tube, pour with 40 ml Broth and insert a 200-ul tip. This tube is for sterility control.
- Put the conical flask and falcon tube into Shaker. Use setting RPM 180-220, 37 ^{0} C. Leave for **10 to 12 hours**. Flask should look cloudy.

Day 03: Setting and Fixation of *Klebsiella pneumoniae* (work inside hood)

- Take a 48 well plate. Use the first row. Mark them as 1/10, 1/100, 1/1000, 10^4, 10^5, 10^6, 10^7 and 10^8.
- Bring the conical flask and falcon tube from the shaker inside hood. Take one 50 ml falcon tube and pour 40 ml in to it. Keep 20 ml for storage purpose.
- Fill the first row with 450 ul sterile and filtered (use round filter to prepare them) PBS. Take 50 ul E coli from the flask and add into first well.
- Mix several times and take 50 ul to add in next well. Mix several times. Do the same for successive wells to get the desired dilution.
- Spin down the falcon tubes (including sterility control) @ 4000 rpm for 8 minutes.
- Remove the supernatant, re-suspend and wash the pallet once in 10 ml of PBS.
- Take out six agar plates from the fridge and level them as 10^4, 10^5, 10^6, $10^{7,}$ 10^8 and sterility control.
- Put 3-4 beads (reusable from PK lab) in each dish and put 50 ul from the corresponding well into petri dish. Use 50 ul sterile PBS for sterility control.
- Manually move all the petri dishes together 8-10 times in each direction (4 directions). Idea is to smoothly spread the bacteria into petri dishes.
- Do not discard the bead; soak them into virkon and autoclave for re-use.
- Keep the petri dishes into designated incubator for 12-16 hours maximum. If it's more than that, the counting of colonies will be difficult as they started to merge and thick.

- Thaw 4% PFA in water bath. Take one-falcon tube and pour with 15 ml in each tube. Add 15 ml sterile PBS in tube to make the composition 2% PFA.
- Add the 30 ml 2% PFA in to previously washed *Klebsiella pneumoniae*. First, put 2-3 ml PFA into tube, mix them with 1000ul pipette, then add the rests.
- Leave at RT for 20 minutes.
- Spin down @ 4000 rpm for 7-8 minutes, remove supernatant, re-suspend and wash with 30 ml sterile PBS for once/ twice.
- Re-suspend in 3 mls filtered sterile PBS (kept on ice). Use 1000ul pipette for accurate measurement instead of 5 ml stripette.
- Keep them in fridge for next day aliquot after counting.

Storage of *Klebsiella pneumoniae* (do it on ice):

- Take 10 one ml cryo vial and level them as ATCC 13368.
- Put 500 ul of *Klebsiella pneumoniae*.
- Add 500 ul Glycerol. Use filtered pipette, as glycerol is very thick and sticky.
- Mix them several times and put into -80 freezer.

Day 04: Counting and preserve

- Take the 10^6 or 10^7 plate for counting colonies. Also, check 10^5 and SC plate. Count the number of colonies (CFU's) carefully. There shall be no colonies in SC plate.
- Multiply the number of colonies (CFU/50 ul) with dilution factor.
- The count will be in million/3000 ul (as total 3 ml).
- Convert them into million/ul.
- Take 500ul in each Eppendorf and store them in fridge for use or -20 freezer for long-term storage.

Annex 16: SOP for *P. aeruginosa* preparation (culture, fixation and counting)

1.0 Reagents and Materials

Item	**Catalog No.**	**Company**
LB Broth, L3022-1 kg	101983727	Sigma Life science
LB Broth with Agar (Lennox), L7025-500 tab	1002414656	Sigma Life science
Glycerol, 500ml	CHE2070	Scientific Lab suppliers
Paraformaldehyde solution 4% (1 litre)	281692	Chem Cruz
Falcon tubes 5ml	734-0442	VWR
Falcon tubes 50ml	352070	VWR
48 well plate flat bottom	353078	Corning Science
Nalgene Polycarbonate Baffled Culture Flasks (500 ml)	4110-0500	Thermo Fisher Scientific

Solutions to be made up **(solutions and plates will be made in CAT II laboratory)**

For Liquid Broth

- Take 10-gram LB powder in a glass bottle.
- Pour with 500 ml miliQ water
- Label it 'LB for growth' and autoclave.
- After autoclave keep in room temperature.

For Agar plate

- Take 500 ml miliQ water in a glass bottle.
- Put 10 LB Agar tablet (1tab/50 ml).
- Label it 'LB for agar plate' and autoclave.
- After autoclave, use 10ml/ Petri dish. Use while hot and clear.
- Wait for 30-60 minutes to cool down and set. Keep them in fridge for future use.

PA Samples

ATCC 27853/ NCTC 12903 strain collected from OU NHS microbiology laboratory.

2. Procedure

Day 01: Setting of colonies in agar plate

- Bring *Pseudomonas aeruginosa* stock from liquid nitrogen and thaw on ice.
- Put the loop into vial and twirl around.
- Streak across the petri dish three times and put into incubator (designated for bacterial growth)

Day 02: Growth of *Pseudomonas aeruginosa* (work inside hood)

- Pour 100 ml Broth into the conical flask (autoclaved).
- Take out the *Pseudomonas aeruginosa* petri dish from incubator and put into fridge 30 mins before collecting colonies.
- Take 10 colonies (1 colony/10 ml) using a 200 ul tip and put it into the conical flask. Wrap the mouth with foil. Keep it little bit loose.
- Take a 50 ml falcon tube, pour with 40 ml Broth and insert a 200-ul tip. This tube is for sterility control.
- Put the conical flask and falcon tube into Shaker. Use setting RPM 180-220, 37 ^{0} C. Leave for **6 to 8 hours**. Flask should look cloudy.

Day 03: Setting and Fixation of *Pseudomonas aeruginosa* (work inside hood)

- Take a 48 well plate. Use the first row. Mark them as 1/10, 1/100, 1/1000, 10^4, 10^5, 10^6, 10^7 and 10^8.
- Bring the conical flask and falcon tube from the shaker inside hood. Take one 50 ml falcon tube and pour 40 ml. keep some for storage purpose.
- Fill the first row with 450 ul sterile and filtered (use round filter to prepare them) PBS. Take 50 ul E coli from the flask and add into first well.
- Mix several times and take 50 ul to add in next well. Mix several times. Do the same for successive wells to get the desired dilution.
- Spin down the falcon tubes (including sterility control) @ 4000 rpm for 8 minutes.
- Remove the supernatant, re-suspend and wash the pallet once in 10 ml of PBS.
- Take out six agar plates from the fridge and level them as 10^4, 10^5, 10^6, $10^{7,}$ 10^8 and sterility control.
- Put 3-4 beads (reusable from PK lab) in each dish and put 50 ul from the corresponding well into petri dish. Use 50 ul sterile PBS for sterility control.
- Manually move all the petri dishes together 8-10 times in each direction (4 directions). Idea is to smoothly spread the bacteria into petri dishes.
- Do not discard the bead; soak them into virkon and autoclave for re-use.

- Keep the petri dishes into designated incubator for 12-16 hours maximum. If it is more than that, the counting of colonies will be difficult as they started to merge and thick.
- Thaw 4% PFA in water bath. Take one-falcon tube and pour with 15 ml. Add 15 ml sterile PBS to make the composition 2% PFA.
- Add the 30 ml 2% PFA in to previously washed *Klebsiella pneumoniae*. First, put 2-3 ml PFA into tube, mix them with 1000ul pipette, then add the rests.
- Leave at RT for 20 minutes.
- Spin down @ 4000 rpm for 7-8 minutes, remove supernatant, re-suspend and wash with 30 ml sterile PBS for once/ twice.
- Re-suspend in 3 mls filtered sterile PBS (kept on ice). Use 1000ul pipette for accurate measurement instead of 5 ml stripette.
- Keep them in fridge for next day aliquot after counting.

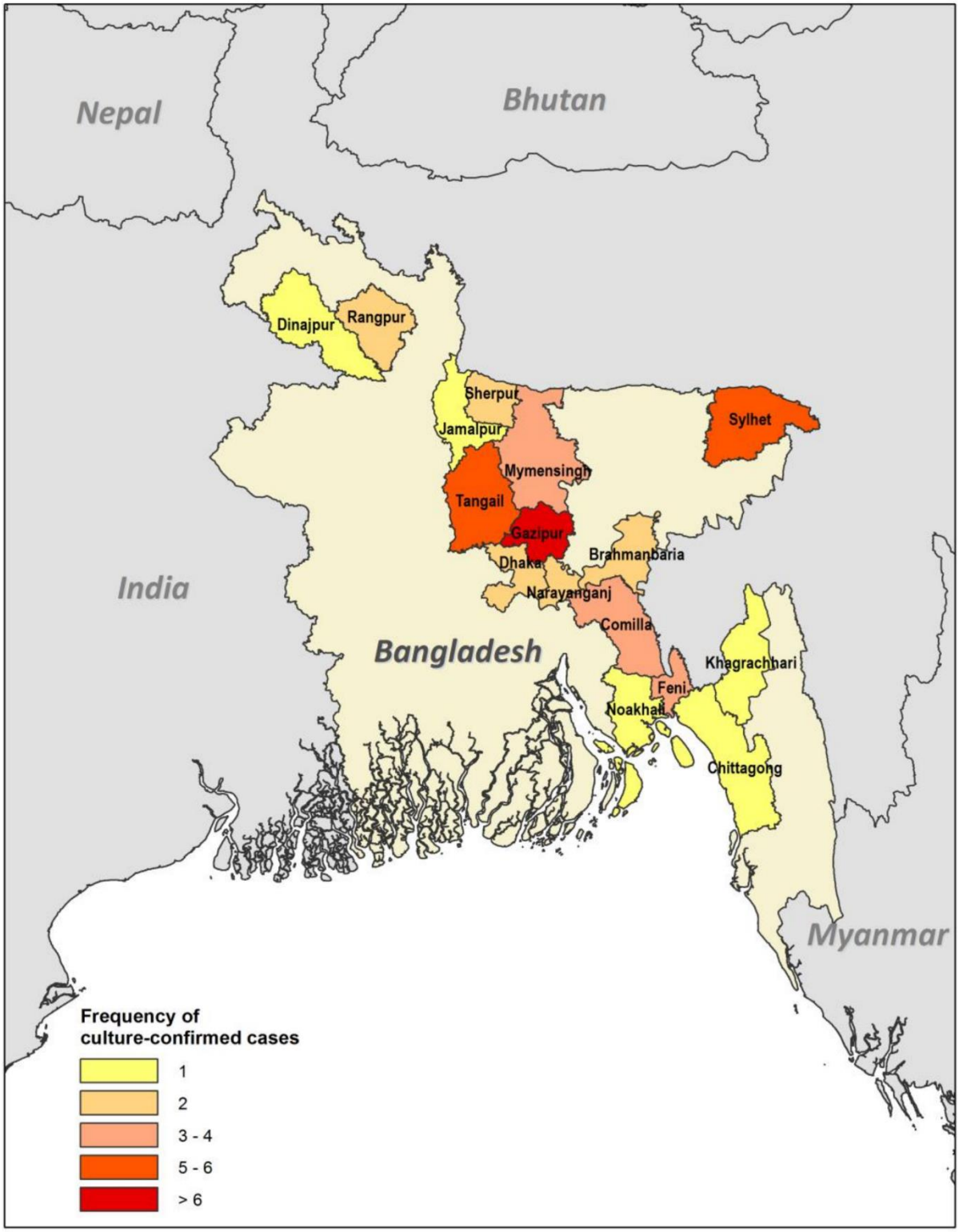
Storage of Pseudomonas aeruginosa (do it on ice):

- Take 10 one ml cryo vial and level them as ATCC: 27853.
- Put 500 ul of *Pseudomonas aeruginosa*.
- Add 500 ul Glycerol. Use filtered pipette, as glycerol is very thick and sticky.
- Mix them several times and put into -80 freezer.

Day 04: Counting and preserve

- Take the 10^6 or 10^7 plate for counting colonies. Also, check 10^5 and SC plate. Count the number of colonies (CFU's) carefully. There shall be no colonies in SC plate.
- Multiply the number of colonies (CFU/50 ul) with dilution factor.
- The count will be in million/3000 ul (as total 3 ml in two tubes).
- Convert them into million/ul.
- Take 500ul in each Eppendorf and store them in fridge for use or -20 freezer for long-term storage.

Annex 17: Choropleth map illustrating the frequency of culture confirmed melioidosis cases by district in Bangladesh



Annex 18: Table: Antimicrobial resistance pattern of all culture confirmed cases (n=21)

Case No	Positive organism	Co-amoxiclav	Ceftriaxone	Co-trimoxazole	Nalidixic acid	Imipenem/ Meropenem	Piperacillin+Tazobactum	Gentamicin	Comment
13	*E. coli*	R	R	R	R	S	S	S	ESBL
78	*E. coli*	N/D	R	S	R	S	N/D	S	ESBL
129	*E. coli*	S	S	S	S	S	S	S	
136	*E. coli*	S	S	S	S	S	S	S	
176	*E. coli*	N/D	R	R	R	S	N/D	N/D	ESBL
114	*E. coli*								
26	*S. aureus*	S	N/D	S	S	N/D	N/D	S	
103	*S. aureus*	R	N/D	R	R	N/D	N/D	R	MRSA
41	*Pseudomonas aeruginosa*	N/D	S	S	S	S	S	R	
139	*Pseudomonas aeruginosa*	N/D	N/D	R	S	S	S	S	
156	*Pseudomonas aeruginosa*	N/D	N/D	R	S	S	S	S	
48	*Acinetobacter sp.*	S	S	S	S	S	S	S	
169	*Acinetobacter sp.*	N/D	N/D	R	R	S	S	R	
60	*S.* Typhi	M	S	S	S	S	N/D	S	
71	*S.* Typhi	N/D	S	S	R	N/D	N/D	N/D	NARST

87	*S.* Typhi	N/D	S	S	R	N/D	N/D	N/D	NARST
147	*S.* Typhi	N/D	S	R	R	N/D	N/D	N/D	MDR Typhoid (resistant to Ampicillin and Chloramphenicol
92	*K. pneumoniae*	N/D	R	R	S	S	N/D	S	ESBL
186	*K. pneumoniae*	R	R	R	S	S	S	S	ESBL
77	Mixed bacterial growth	-	-	-	-	-	-	-	Micrococcus (possible contamination)
94	Mixed bacterial growth	-	-	-	-	-	-	-	Possible contamination
152	Mixed bacterial growth	-	-	-	-	-	-	-	Possible contamination

N/D: not done, S: sensitive, R: Resistant, M: Moderately sensitive