Establishing two systems to study MAIT cell responses during bacterial infection

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Abstract

Mucosal-associated invariant T (MAIT) cells are a population of innate-like T cells, which are abundant in humans and also present in many mammals, including mice [1]. MAIT cells express semi-invariant T cell receptors (TCR) are activated by recognizing microbial vitamin B2 (riboflavin) metabolites presented on non-classical major histocompatibility complex (MHC) class I related protein 1 (MR1) [1-6]. Upon activation, MAIT cells can either directly kill infected cells or secrete IFN-γ, IL-17, TNF and other functional molecules that enhance the anti-microbial activity of other immune cells [7, 8]. These characteristics suggest an important role played by MAIT cells in anti-bacterial immunity in mammals.

Bacterial infections are major problems in clinical settings. While MAIT cells have shown antibacterial properties, clinically relevant *in vivo* animal models have not been extensively developed for further exploration of the mechanisms of MAIT cell protection. Using three clinically common bacteria; *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli*, this study established bacterial peritonitis models in mice for MAIT cell research. The three bacteria were all able to produce antigens that stimulate MAIT cells *in vitro.* For mouse infection models, the optimal dose of *K. pneumoniae* for inducing productive but nonlethal infection was not established in this study. *S. aureus* and *E. coli* peritonitis incurred mild and robust MAIT cell responses *in vivo*, respectively. Further experiments with *E. coli* peritonitis showed that MAIT cells accumulated in the intraperitoneal cavity, the spleen and liver, which contributed to bacteria control. The finding suggests possible MAIT cell-based treatments in clinical conditions.

While *in vitro* studies have shown that MAIT cells can be stimulated by various types of cells upon infection, there have been few investigations into how MAIT cells are activated *in vivo*. In the second project presented in this thesis, the contributions of antigen presenting cells (APCs) in initiating activation in MAIT cells was studied with *Francisella tularensis* infection. We found that APCs were burdened with bacteria in the early phase of infection. With an *ex vivo* cellular co-culture assay was specifically optimized for assessing MAIT cell activation induced by the *F. tularensis* burdened APCs, we found that alveolar macrophages contributed to the best activation of MAIT cells in an MR1 dependent manner. Other cell subsets: monocytes, neutrophils and dendritic cells were also capable of inducing a mild activation.

In summary, the present study helped optimize two novel systems for MAIT cell research. With the *ex vivo* cellular co-culture system, this thesis contributes to

understanding how APCs activate MAIT cells *in vivo* upon bacterial infection. With the *in vivo* peritonitis models, this study introduces clinically relevant bacteria to the current MAIT cell research in mice, and ultimately to inform and complement clinical research. It is hoped that the insights gained from this study could be of assistance to current research methods and to achieve a better understanding of MAIT cell responses during bacterial infection.

Declaration

This is to certify that:

1) The thesis comprises only my original work towards the Master of Research except where indicated in the preface;
2) Due acknowledgement has been made in the text to all other material used;
3) The thesis is fewer than 50,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signature: _____ Date: _____

Tianyuan Zhu

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Abbreviations

APC	Antigen presenting cell
CD	Cluster of differentiation
DP	Double positive ($CD4^+$ $CD8^+$)
DN	Double negative ($CD4^-$ $CD8^-$)
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
IFN	Interferon
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
LVS	Live Vaccine Strain of *Francisella tularensis*
mAb	Monoclonal antibody
MAIT	Mucosal-associated invariant T
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
RPMI	Roswell Park Memorial Institute
RORγt	RAR-related orphan receptor gamma
PBS	Phosphate buffered saline
PLZF	Promyelocytic leukemia zinc finger
PMA	Phorbol 12-myristate 13-acetate
SPF	Specific pathogen-free
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
5-OE-RU	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
5-A-RU	5-amino-6-D-ribitylaminouracil
6-FP	6-formylpterin

7-AAD	7-aminoactinomycin D
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-cyanine7
FITC	Fluorescein isothiocyanate
FVD	Fixable Viability Dye
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cyanine7
PerCP-Cy5.5	Peridinin chlorophyll protein-cyanine-5.5

Contents

CHAPTER 1: LITERATURE REVIEW 12

1.1 The immune system 12

1.1.1 Innate immunity 12

1.1.2 Adaptive immunity 13

1.1.2.1 T cells 15

1.1.2.2 Unconventional T cells 18

1.2 MAIT cells: a relatively new population of innate-like T cell 19

1.2.1 MAIT cell receptors, phenotype and tissue tropism 20

1.2.2 MR1 and antigens 21

1.2.3 Activation of MAIT cells 23

1.3 MAIT cell in bacterial infection diseases 25

1.3.1 Human studies 25

1.3.2 Mouse studies 27

1.4 Research aims 30

CHAPTER 2: MATERIAL AND METHODS 32

2.1 Material: reagents and buffers 32

2.2 Methods 34

2.2.1 Ethics 34

2.2.2 Bacteria and growth conditions 34

2.2.3 Bacterial supernatant and lysate preparation 36

2.2.4 Bacterial infection to mice 36

2.2.5 Assessment of bacterial load by CFU analysis 36

2.2.6 Preparation of single cell suspension from organs 37

2.2.7 Fractionation of lymphocytes using Percoll 37

2.2.8 Surface and intracellular staining 38

2.2.9 Flow cytometry and cell sorting 40

2.2.10 Ex vivo activation assay 42

2.2.11 Cell lines and activation assay 43

2.2.12 Statistical Analyses 43

CHAPTER 3: ESTABLISHING PERITONITIS MODELS FOR ASSAYING MAIT CELL ROLES USING BACTERIA REPRESENTATIVES OF THOSE MOST FREQUENTLY FOUND BACTERIA CLINICALLY. 44

3.1 Introduction 44
3.2 Results 46

3.2.1 Growth kinetics of Klebsiella pneumoniae, Staphylococcus aureus and Escherichia coli. *46*
3.2.2 K. pneumoniae, S. aureus and E.coli produce antigens that activate a MAIT reporter cell line. *48*
3.2.3 K. pneumoniae fails to induce a productive but non-lethal intraperitoneal infection in C57BL/6 mice. *51*
3.2.4 S. aureus intraperitoneal infection causes local MAIT cell accumulation *53*
3.2.5 E.coli ST131 infection induces a significant accumulation of MAIT cells locally and in peripheral organs. *56*
3.2.6 MAIT cell deficient Mr1$^{-/-}$ mice are more susceptible to E. coli peritonitis. *60*
3.2.7 MAIT cells are skewed to a MAIT-1 (Th1-like) phenotype during E. coli infection *62*

3.3 Discussion 68

CHAPTER 4: DEFINING ANTIGEN PRESENTING CELL SUBSETS FOR MAIT CELL ACTIVATION DURING *FRANCISELLA TULARENSIS* INFECTION 72

4.1 Introduction 72
4.2 Results 73

4.2.1. Bacteria were barboured by APCs after F. tularensis LVS intranasal infection of mice. *73*
4.2.2 Trouble shooting for ex vivo MAIT cell activation assay. *77*
4.2.3 APCs, especially alveolar macrohages, activate MAIT cells in an MR1 dependent manner when burdened with F. tularensis LVS. *80*
4.2.4 MAIT cell activation by AM is blockable by anti-MR1 antibodies. *85*

4.3 Discussion 87

CHAPTER 5: GENERAL DISCUSSION 90

5.1 Peritonitis as a novel infection model for MAIT cell studies 90
5.2 *Ex vivo* assay as a new system for unravelling MAIT cell responses *in vivo* 92
5.3 Conclusion of the thesis 94

REFERENCES..... 95

List of figures

Figure 1 Antigen presentation to different T cell subsets......16

Figure 2 Activation of MAIT cells during infection......24

Figure 3 Gating strategy to identify murine MAIT cells......41

Figure 4 Growth kinetics of *K. pneumoniae*, *E. coli* and *S. aureus*......47

Figure 5 Bacterial supernatant activated Jurkat.MAIT cells *in vitro* as assessed by CD69 expression......49

Figure 6 Bacterial lysate activated Jurkat.MAIT cells *in vitro* as assessed by CD69 expression......50

Figure 7 Weight change of mice following intraperitoneal infection with *Klebsiella pneumoniae.*.....52

Figure 8 Bacterial load kinetics of C57BL/6 mice during *S. aureus* BPH2819 intraperitoneal infection......54

Figure 9 Infection of clinical strain of *S. aureus* led to MAIT cell expansion in the abdominal cavity, but not in liver or spleen......55

Figure 10 Body weight and bacterial load kinetics of C57BL/6 mice during *E. coli* infection......57

Figure 11 Representative FACS plots demonstrate *E. coli* infection boosted MAIT cells in affected organs......58

Figure 12 *E. coli* ST131 intraperitoneal infection caused significant accumulation of MAIT cells in the peritoneal cavity, liver, spleen and kidney......59

Figure 13 *E. coli* peritonitis was less well controlled in MAIT cell deficient *Mr1*$^{-/-}$ mice......61

Figure 14 Transcription factor profiling of MAIT cells during acute *E. coli* ST131 peritonitis......64

Figure 15 Cytokine profile of MAIT cells during acute peritonitis by *E. coli* ST131. 67

Figure 16 Gating strategy to identify lung APC subsets. 74

Figure 17 The percentage of APCs increased after *F. tularensis* LVS intranasal infection. 75

Figure 18 Bacterial load varied in different APC subsets after *F. tularensis* LVS lung infection. 76

Figure 19 MAIT cells boosted *in vivo* by *F. tularensis* LVS or *L. longbeachae* differ in CD69 expression. 79

Figure 20 Schematic of *ex vivo* system assaying MAIT cell activation by *F. tularensis* LVS infected APCs. 81

Figure 21 After boosting *in vivo*, $CD69^+$ MAIT cells did not further upregulate CD69 expression following *ex vivo* stimulation. 83

Figure 22 $CD69^-$ MAIT cells were activated after co-cultured with lung APCs from *F. tularensis* LVS infected mice. 84

Figure 23 Lung APCs from *F. tularensis* LVS infected mice activated MAIT cells *ex vivo* in an MR1-dependent manner. 86

List of tables

Table 1 Characteristics of CD4$^+$ effector T cell subsets {Murphy, 2012 #350}. 18

Table 2 Established mouse models studying MAIT cell response upon bacterial infection. 28

Table 3 Reagents and buffers. 32

Table 4 Strains of bacteria that were used in this study with culture conditions. 35

Table 5 Formula of preparing Percoll working solution. 37

Table 6 Staining panel of murine MAIT cells. 38

Table 7 Staining panel of APCs in the mouse lung. 39

Table 8 Staining panel of cytokines and transcription factors of mouse cells. ..40

Table 9 Staining panel of *ex vivo* activation assay. 42

Table 10 Staining panel of *in vitro* activation assay. 43

Table 11 Causative microorganisms of spontaneous bacterial peritonitis and secondary peritonitis 45

Chapter 1: Literature Review

1.1 The immune system

The immune system of mammals is a complex and integrated system of defence, consisting of immune organs, tissues, cells and molecules, which work together to resist pathogen invasion in either antigen nonspecific (innate) or specific (adaptive) manner [9]. Generally, innate immunity is rapidly responsive and protects mammalian hosts during the early stage of infection, while adaptive immunity requires a longer time for preparation but attacks microbes with higher specificity [9]. Additionally, adaptive immunity has the ability to generate specific memory that provide long-lasting protections upon subsequent infection. Therefore, the immune system defends the infectious pathogens efficiently and in hierarchy.

1.1.1 Innate immunity

Innate immunity provides an always "on" or at least quick responsive mode of protection [10] which is at the frontier of confronting external pathogens or internal stresses. There are two layers of protection offered by innate immunity. The first line of defence is composed of physical barriers, including skin, epithelial, mucosa, excreted body fluids and normal flora, which block the pathogens from entering the body [11]. If the harmful agent manages to invade, the second line of defence is mobilized to prevent and clear the infection. This second line consists of effector molecules such as complement and cytokines, and many types of innate immune cells, including macrophages, neutrophils, basophils, eosinophils, mast cells, dendritic cells (DCs), natural killer (NK) cells and innate lymphoid cells (ILCs) [12]. These immune cells patrol the body at all times and once they detect pathogenic agents, they become activated to clear the pathogens by a variety of mechanisms (reviewed in [9]).

Generally, the activation of innate immune cells depends on recognizing pathogen-associated molecular patterns (PAMP) [13, 14] or damage-associated molecular patterns (DAMP) [15], which are conserved molecular patterns wildly present in pathogens or injured tissue but not in any normal self-structure. The innate immune cells recognize PAMPs/DAMPs using a group of evolutionarily conserved pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin

receptors (CLRs), scavenger receptors (SR) and NOD like receptor (NLR) [16-19]. When PAMPs/DAMPs are recognized, they are captured and bind to the PRRs expressed by those innate immune cells and this process leads to the activation of them [20].

Upon activation, innate immune cells can destroy pathogens or respond to tissue damage in distinctive ways. Phagocytic cells including macrophages and neutrophils internalize bacteria to endocytic vacuoles, where the bacteria are killed by acidification and infusion of lysosome [21]. Phagocytes can also produce an array of cytokines and chemokines, thus recruiting other immune cells to the site of infection [22]. In contrast, basophils, eosinophils and mast cells are capable of releasing mediators, a family of compounds that induce local inflammation which is not suitable for microbial growth. NK cells are characterized by their cytolytic effect, and they control viral infection by inducing apoptosis in infected cells [23].

When the invaded pathogens persist, adaptive immunity is also mobilized upon signals provided by specific innate antigen presenting cells (APCs), especially dendritic cells [24]. Dendritic cells capture microbe and present the processed antigens using a range of surface molecules, of which major histocompatibility complex (MHC) is one of the most important groups [25, 26]. Dendritic cells migrate to peripheral lymphoid organs such as lymph nodes [25, 26], where the adaptive immune cells reside, and trigger adaptive T cell immune responses by presenting the antigen loaded on MHC molecules [27].

1.1.2 Adaptive immunity

Adaptive immunity is characterized by its high specificity compared to innate immunity. B and T cells, which derive from bone marrow and thymus respectively, are two main players of adaptive immune responses. The specificity lies in the antigen-specific and highly polymorphic surface receptors of T and B lymphocytes, namely T cell receptors (TCRs) and B cell surface immunoglobulins (sIgs), also known as B cell receptors (BCRs) . Generated by rearrangement of TCR and Ig gene segments, sIgs and TCRs are highly diverse (10^{11}) [28-30] and thus enable recognition of a diverse array of foreign antigens [29]. An adaptive immune response initiates when B and/or T cells recognize their cognate antigens with surface sIgs and/or TCRs. Two types of adaptive immune response, namely cell mediated and antibody (humoral) mediated immune responses, are provided by T and B lymphocytes respectively. The

antibody mediated immune response (humoral response) is triggered when naïve B cells recognize antigens either unbound or bound to follicular dendritic cells [31]. Upon this antigen-driven recognition, B cells are activated and undergo clonal expansion as well as differentiation, allowing the generation of large amount of effector B cells that recognize this particular antigen. Effector B cells (plasma cells) produce large amounts of circulating antibodies that specifically bind to cognate antigens of the pathogens, which either neutralize microbes directly or label them for further destruction [32]. Cell mediated immune response is triggered when naïve T cells recognize antigens presented by migrated dendritic cells and become activated. The effector T cells, e.g. cytotoxic T cells (T_C), are clonal expanded progenies of activated T cells, therefore inherit a similar specificity to the particular antigen [27]. These cells specifically recognize and eliminate infected body cells by either stimulating apoptosis cascade or releasing perforin, a protein that disrupts cell membrane and induce cell lysis [33].

Despite their distinct functions, T and B cells also rely on each other to achieve a fine-tuned adaptive immunity. Apart from cytotoxic T (Tc) cells, there are also considerable amount of T helper cells (Th) that provide the essential co-stimulatory signals for B cell activation [34, 35]. B cells can also act as antigen presentation cells for T cell activation [36].

In addition to its high level of specificity, adaptive immunity can also "remember" specific microbes and provide lasting protection, a process known as immunological memory. During primary infection, while most activated B and T cells differentiate into effector cells and die after pathogens resolved, small populations of memory cells are retained. These memory cells provide an enhanced and still specific response upon subsequent challenges [9, 37]. Vaccination takes advantage of immunological memory, to immunize people against fatal infections with pathogens [38, 39].

Despite their differences and independency, the innate and adaptive immunity are not "poles apart", but work in synergy to establish a solid defence system [40]. Adaptive immune responses depend on innate signals for initiation, and in reverse, enhance innate immune responses. Overall, it is essential that every component of the immune system plays its part precisely to resist external and internal stresses, and diseases arise when immunity fails to do so. To date, there are still many gaps in studying the incredibly complex immune system.

1.1.2.1 T cells

T cell progenitors are formed in the bone marrow, become mature T cells in the thymus and migrate to periphery [9]. As discussed previously, T cells are main players of adaptive immune responses and are characterized by the cell surface expression of TCR complex, which recognizes cognate antigen presented by APCs [27]. A TCR complex contains an invariant CD3 molecule as well as a heterodimer of an α and β chain, or of a γ and δ chain, which is expressed respectively on αβ T cells or less abundant γδ T cells [9]. During thymic development of T cells, genes encoding TCR-α and TCR-β chains are generated by somatic rearrangement, leading to a total diversity of 10^{18} for gene segments [9, 29]. Thus, αβ TCRs are highly polymorphic, allowing the recognition of various antigens. By contrast, γδ TCRs have a limited diversity, hence recognize limited antigens. Moreover, T cells recognise antigens presented by different surface molecules of APCs, including the highly polymorphic classical MHC molecules and non-classical molecules such as CD1d and MHC class I related protein 1 (MR1) (Figure 1), contributing to even greater diversity in the range of molecules that can be detected [6, 9, 41]. The recognition of different classes of antigen, presented on different antigen-presenting molecules defines several subsets of T cells and among which αβ T cells, whose activation is restricted to MHC molecules, have been best described [9, 42]. These conventional T cells recognize peptide antigens and are also known as $CD8^+$ and $CD4^+$ T cells due to expression of co-receptor CD8 and CD4, which assist T cell binding to MHC class I and II molecules respectively [43]. Other T cells, including natural killer T (NKT) cells, γδ T cells and mucosal-associated invariant T (MAIT), cells are restricted by non-classical MHC molecules and recognize non-peptide antigens, therefore are classified as unconventional T cells [44, 45]. They are also known as innate-like T cells due to the display of some innate cell markers and innate functional features.

It is generally believed that conventional T cell display naïve features when become mature and exit from thymus [9]. During an immune response, naïve T cells first encounter their cognate antigens presented by DCs [26, 27]. Consequently, naïve T cells undergo priming and expansion in the lymphoid organs to become effector T cells, which possess and fulfil enhanced immune functions such as clearance of pathogens [9]. In the infection resolving phase, a small proportion memory cells are retained, which mount a quicker (no priming stage needed) and stronger immune response upon subsequent infection of a same pathogen [46]. Unconventional, or innate-like T cells, confer innate like and rapid immune responses but do not form immunological memory [47].

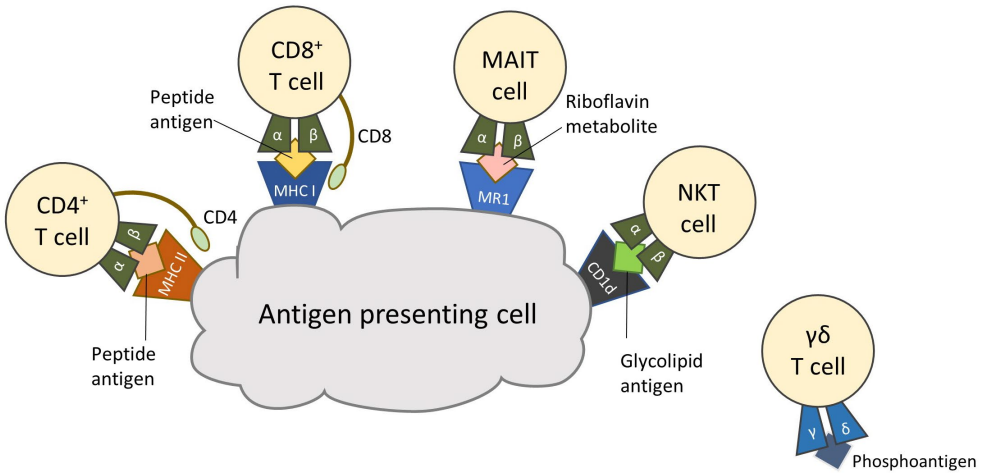


Figure 1 Antigen presentation to different T cell subsets.

$CD4^+$ and $CD8^+$ T cells express conventional αβ TCRs, which recognize peptide antigens presented by major histocompatibility complex (MHC) class II and class I molecules respectively. Their bindings of TCR with antigen bound MHC molecules class II and class I is assisted by co-receptor CD4 and CD8. Mucosal-associated invariant T (MAIT) cells and natural killer T (NKT) cells express different semi-invariant αβ TCRs. MAIT cells recognize riboflavin metabolites presented with MHC-related protein 1 (MR1). NKT cells recognize glycolipid antigens presented with CD1d. γδ T cells express less invariant γδ TCRs and recognize phosphoantigens, small peptide and soluble proteins, but the mechanisms mostly remain unknown [48].

$CD8^+$ T cells are also known as cytotoxic (Tc) cells that recognize antigens presented by MHC-I, a surface protein expressed on every single body cell other than erythrocytes. When body cells are infected with intracellular pathogens, MHC-I molecules present processed pathogenic peptides, which make Tc cells recognize the infected cells [49]. The interaction between antigen MHC-I complex and TCRs primes Tc cells, and after receiving co-stimulatory signals provided by $CD4^+$ T cells [50], primed Tc cells are fully activated [9]. These cells then clonally expand their population and differentiate into antigen-directed effector Tc cells, who which recognize more infected cells by the membrane-bound antigen MHC-I complex and thereafter, destroy the infected cells. Specifically, effector Tc cells manifest their function by releasing an array of cytotoxic proteins including perforin, a protein that forms pores in the membrane of target cells, and granzyme, a protease that enters target cells through the pores and initiates apoptosis cascades leading to cell death. Tc cells also express Fas ligands (FasL, or Apo1L, CD95L) which deliver apoptosis signals after binding to the Fas receptors (Apo1, CD95) on the surface of target cells [33]. Tc cells secret cytokines too, which help to recruit or modulate function of other immune cells [51, 52].

Although cytotoxic capacities were also found in some $CD4^+$ T cells [53], most $CD4^+$ T cells are not cytotoxic but are important accessory cells in adaptive immunity, thus are generally termed as helper T cells (Th). Th cells recognize cognate antigens presented by MHC-II molecules, whose expression is restricted professional APCs cells, including macrophages, dendritic cells, B cells and some nonprofessional APCs such as specialised epithelial cells. The interaction of TCR and antigen MHC-II complex delivers activation signals to naïve Th cells, which consequently differentiate into effector Th cells and expand clonally.

According to the signals (i.e. cytokine) provided by the priming antigen presenting cells and the particular milieu condition under inner and outer stresses, naïve $CD4^+$ T cells can differentiate into different subsets of effector cells [54]. These subsets produce distinctive cytokines which regulate and assist other immune cells to conduct their functions (Table 1).

Table 1 Characteristics of $CD4^+$ effector T cell subsets (modified from reference [55]).

	Inducing cytokine	Transcription factor	Cytokine production	Target cells
Th1	IL-12, IFN-γ	STAT4, T-bet	IFN-γ, TNF	Macrophage, T_C, IgG B cell
Th2	IL-4	STAT6, GATA3	IL-4, IL-5, IL-13	Mast cell, eosinophil, basophil, IgE B cell
Th3	TGF-β, IL-4	Foxp3	TGF-β, IL-10	B cell
Th9	TGF-β, IL-4	PU.1/Spi-1, IRF4	IL-9 (human), IL-10 (mouse)	T cell, IgG B cell, mast cell, epithelial cell
Th17	TGF-β, IL-6	STAT3, RORγt	IL-1, IL-6, IL-17, TNF-α	Neutrophil
Th22	IL-6, TNFα	AHR	IL-22, IL-13, TNFα	Epithelial and stromal cell
Thαβ	IFN-α/β	STAT1, STAT3, IRF	IL-10	NK cell
Tfh	IL-6, IL-21	Bcl-6	IL-21	B cell
Treg	TGF-β	Foxp3	TGF-β	Effector T cell, APC

The most well-known $CD4^+$ T cell subsets include Th1, Th2, Th17 and Treg, which are distinctive in their phenotype and pattern of response. Th1 cells are professionals in assisting the activation of macrophage by secreting signature cytokines such as interferon gamma (IFN-γ) and tumour necrosis factor (TNF) [56]. Th2 cells mainly help B cells to produce monoclonal antibodies. Th17 cells are characterized by their production of IL-17 [57], which helps recruiting neutrophils and stimulating chemokine production in other somatic cells. Treg cells suppress the activity of immune cells such as T cells and dendritic cells to avoid overreaction of immunity [58], while they are also responsible for chronic infection and immune escape of tumour cells [59, 60].

1.1.2.2 Unconventional T cells

Apart from $CD4^+$ and $CD8^+$ T cells, there are many other types of T cells defined by their conserved, less variant TCRs compared to the conventional αβ TCRs. These unconventional T cells, including γδT cells, NKT cells and MAIT cells, are restricted to non-classical MHC molecules during ontogeny and recognize antigens other than

peptide [1, 4, 61-63] (Figure 1). Therefore, unconventional T cells are believed to be connective cells between innate and adaptive immunity.

γδT cells undergo thymic development similar with αβT cells, but alternatively, generate γ and δ heterodimer TCRs and become $CD4^{-}CD8^{-}$ [64]. γδ TCRs are far less antigen-specific but resembles PRR in recognizing conserved patterns of pathogens [65], therefore define the unconventional properties of γδT cells. Homing preferentially in mucosal and epithelial tissues, γδT cells have been found to display wide functional plasticity and suggested to assist early defence against infection and internal stresses [45, 66]. However, the detailed mechanism of γδ T cells responses are still largely unknown.

NKT cells share defining receptors of both NK cells (NK1.1) and T cells (TCR), but they express semi-invariant αβ TCRs hence have a more limited TCR repertoire hence express semi-invariant αβ TCRs [61]. NKT cells are restricted to non-polymorphic CD1d receptors during ontogeny and after migrating to peripheral, can be stimulated by CD1d loaded with glycolipid or phospholipid ligands from external or internal sources [67, 68]. According to the expression of TCRs, NKT cells are classified as type 1 invariant NKT (iNKT) cells, which bear invariant TCR α chains (Vα14Jα18) and type 2 NKT cell which have more diverse TCRs. The best described subset is iNKT cells, also known as classical NKT cells, that rapidly respond to both self and non-self signals [69]. Although iNKT cells are rare in human, constituting 0.0001-1% of circulating $CD3^{+}$ cells [44, 70], they have been reported to be important regulators of immune responses upon infection, tumour and autoimmunity [71].

1.2 MAIT cells: a relatively new population of innate-like T cell

Mucosal-associated invariant T (MAIT) cells were originally described by Tilloy *et al.* in 1999 [1] before being named by Treiner *et al.* in 2003 [2], and considerable literature has been published in the 20 years since. Conserved among mammalian species, MAIT cells are an abundant population of T cells in human adults but rare in neonates and laboratory strains of mice [44, 72, 73].

MAIT cells express various membrane bound receptors, including TCRs, co-receptors and an array of cytokine and chemokine receptors. Different from endogenous T cells, MAIT cells express semi-invariant αβ TCRs which specifically recognize a new class of antigen, vitamin B metabolites [1, 4, 5]. These antigens are produced by many

bacteria and fungi but not humans or other mammals [5], therefore can be distinguished by MAIT cells as non-self structures. In addition, the antigens are presented by MHC-I related protein 1 (MR1) molecule, an invariant and conserved protein ubiquitously expressed by all types of cell [74]. The recognition of antigen-MR1 complex will induce activation in MAIT cells, and consequently, lead to release of cytokine and cytotoxic molecules by MAIT cells [7].

Given the conservation of MR1 in mammals and wild presence of MAIT ligands in microbes, it was hypothesized and is now clear that MAIT cell is an important component of antimicrobial immunity [75, 76]. Additionally, literature also suggest the participation of MAIT cells in several non-infectious diseases, including autoimmune diseases [77-81] and malignant diseases [81]. These findings have emphasized the importance of innate-like MAIT cells and brought more insight into the innate and adaptive immunity.

1.2.1 MAIT cell receptors, phenotype and tissue tropism

MAIT cells are most characterized by their unconventional, semi-invariant TCRs. In 1999, Tilloy *et al.* first identified the TCR α chain of MAIT cells, containing TRAV1-2- TRAJ33 (Vα7.2-Jα33) in humans and the homologous AV19-AJ33 (Vα19-Jα33) in mice [1]. Further research has described that MAIT cell TCRs are composed of semi-invariant α chains TRAV1-2- TRAJ33/12/20 (Vα7.2-Jα33/12/20) with β chains from a limited repertoire, which include TRBV20 (Vβ2) and TRBV6 (Vβ13) in human, Vβ6 and Vβ8 in mice [72, 82]. The expression of co-receptors in MAIT cells is also distinct from that in conventional T cells. In humans, MAIT cells in peripheral blood are mostly (~95%) $CD8^+$ single positive (SP) or $CD4^-CD8^-$ double-negative (DN), but rarely $CD4^+$ single positive [73]. In contrast, murine MAIT cells are mostly DN, while the percentage of $CD8^+$ and $CD4^+$ MAIT cells vary with the organs or tissues the MAIT cells reside in [83].

In adult humans and mice, MAIT cells exhibit an effector memory phenotype ($CD45RA^{lo}$ $CD45RO^+CD95^+CD27^+CD122^+$ in humans, $CD44^{hi}CD62L^{lo}$ in mice) [72, 84-86], expressing the transcription factors promyelocytic leukemia zinc finger (PLZF) and retinoic acid–related orphan receptor γt (RORγt) [72, 83, 87]. In addition, CD161, a surface marker of NK cells and also suggesting memory phenotypes in circulating T cells [72, 84, 88, 89], is highly expressed in human MAIT cells. However, the counterpart of CD161 has not been found in mice [90]. A definitive receptor for

both human and murine MAIT cells is interleukin (IL)-18Rα [75, 90], while several other cytokine receptors, including IL-7Rα and IL-12Rβ, are also expressed by MAIT cells [75, 91]. Additionally, human MAIT cells from peripheral blood express a series of chemokine receptors, specifically CCR5 (lung), CCR6 and CXCR6 (liver), CCR9 (intestine) but not CCR7 (lymph nodes) [72, 85], suggesting their tissue-homing properties. This is roughly consistent in mouse MAIT cells, which are $CCR6^{+}$ $CCR7^{-}$ but $CCR5^{-}$ [90].

Humans have a scarcity of MAIT cells in the thymus and cord blood [1, 72], but MAIT cells expand to reach high numbers after birth [1, 92] and become abundant in adults. As indicated by their name, MAIT cells preferentially reside in the mucosal tissues, which is also where infections frequently occur. In human adults, the percentage of MAIT cells is 3-7% of total T cells in intestinal mucosa [84, 93], 0.1-7% in the buccal mucosa [94], 20-40% in liver [72]. Also MAIT cells are present in the mesenteric lymph nodes [84, 93], lung [95, 96] uterine mucosae [97] and circulatory system, constituting 1-10% of the T cell population in the peripheral blood [72, 75]. Upon local infection, MAIT cells can further accumulate at the infected tissues [83, 95, 98], leading to a drop of frequency in peripheral blood [99, 100].

Unlike in humans, MAIT cells are rare in classical laboratory strains of mice (~0.1% in lymphoid organs) but are distributed similarly, compared to humans MAIT cells [1, 72, 82]. In C57BL/6 mice, MAIT cells comprise around 0.7% of total αβ T cells in the intestinal lamina propria, 0.4-2% in lungs [101], 1-5% in the liver [83, 90], 2% in female genital tract [73, 95]. Additionally, a 20-fold higher expression of MAIT iTCR was found in naïve CAST/Ei mice, which were from a wild derived inbred strain [90].

1.2.2 MR1 and antigens

The development of MAIT cells during ontogeny is restricted to MR1. As MHC molecules to conventional T cells, MR1 is required for antigen presentation to MAIT cells [2]. MR1 is a non-polymorphic molecule that is highly conserved in mammals, as human and murine MR1s share remarkably (90%) homology in gene sequences and cross-reactivity in functions [3, 75, 102]. Although its gene expression has been detected widely [3, 103], MR1 largely stays intracellularly in a semi-folded conformation during steady states, with only 5% of MR1 molecules expressed on the cell surface [104]. However, surface MR1 is significantly upregulated when a ligand bind to MR1 by forming a covalent bond (Schiff base), which assists MR1 folding

and induce the transportation of MR1-antigen complex to the cell surface [104]. Based on this "presentation on demand" fashion, MR1 actively presents antigens to MAIT cells specifically upon the presence of exogenous ligand [104].

MR1 has been defined as early as 1995 [105], and its relationship with MAIT cells has been well described before 2005 [2, 6]. However, it took another 7 years to successfully reveal the first ligand of MR1 molecules. The first discovery of MR1 ligand started from an interesting finding of MR1 refolding in cell culture medium, suggesting the presence of antigen that stabilized MR1 in the medium [4]. This antigen was further determined to be 6-formylpterin (6-FP), a folic acid (vitamin B9) metabolite which binds to MR1 but cannot stimulate MAIT cells [4]. Other folate-based antigens such as acetyl-6-FP (Ac-6-FP) have also shown non-stimulatory properties for MAIT cells [5]. Following a similar method, the stimulatory ligands of MAIT cells were characterized to be riboflavin (vitamin B2) metabolites[4]. An intermediate compound of riboflavin biosynthesis, 5-Amino-6-D-ribitylaminouracil (5-A-RU), has been identified as the precursor of two highly activating ligands of MAIT cells, 5-(2-oxopropylideneamino)-4-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) [5]. Through a non-enzymatic reaction, 5-OP-RU and 5-OE-RU can be formed by adding small molecules, methylglyoxal and glyoxal respectively, to 5-A-RU [5]. Riboflavin is an important component of microbial metabolism, and thus, its biosynthetic pathway is widely present in bacteria and yeast, including *Salmonella* Typhimurium, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, Mycobacterium spp., *Legionella* spp., *Candida* spp. and *Aspergillus* spp. [106, 107]. Given that mammals cannot produce riboflavin, detecting ligands derived from the riboflavin biosynthesis pathway is an ideal way for MAIT cells to detect microbial infections.

The discovery of MR1 ligands has promoted MAIT cell study, not only by unravelling the molecular details of MAIT cell antigen recognition, but also by the generation of MR1-5-OP-RU tetramers, which allow specific detection of MAIT cells. Before the MR1-tetramers were developed, MAIT cells were most commonly defined as a $CD3^{+}V\alpha7.2^{+}CD161^{hi}$ lymphocyte population in humans, but the antibody based staining panel using these and other surrogate markers is imprecise due to the CD161 expression shared by other T cells and NK cells [108]. The circumstance was even worse for studying murine MAIT cells, with the technical difficulties in generating Vα19 antibody and no CD161 equivalent found in mice, besides the rarity of MAIT cells in most experimental mouse strains. By tetramerizing four MR1 molecules loaded with 5-OP-RU, MR1-5-OP-RU tetramers have provided a further confirmation

of MR1 restriction to facilitate the MAIT cell detection with higher specificity [5, 73, 83]. The tetramer can also be tagged with fluorescent dyes, thus marks the tetramer^{+} MAIT cells for flow cytometry analysis. Utilizing this new tool, recent studies have revealed a ~90% consistency between tetramer^{+} and CD3^{+}Vα7.2^{+}CD161^{hi} T cell populations in humans [73]. Also, MAIT cells were detected by tetramer in most organs of naïve mice [83].

1.2.3 Activation of MAIT cells

To date, studies have demonstrated two mechanisms of MAIT cells activation, which are TCR-dependent and TCR-independent (cytokine-dependent) (Figure 2). As reviewed in the previous section, MAIT TCRs recognize riboflavin metabolites presented by MR1. Therefore, microbes possessing a riboflavin biosynthesis pathway are generally required to initiate a TCR-dependent response of MAIT cells during infection [75, 109]. Moreover, the activation is abolished when MR1 molecules of APCs were blocked by antibodies [75], suggesting that MR1 is essential in inducing the MAIT cell response.

MAIT cells can also be stimulated in a TCR-independent manner. In this circumstance, the activation of MAIT cells does not require an antigen presentation process but rather to be induced by cytokines. In particular, IL-12 and IL-18 stimulations are most reported, and MAIT cells are found activated in response to recombinant cytokines with or without other danger signals (i.e. Toll-like receptor agonist) [91, 110]. Also, IL-18 has been suggested crucial in triggering MAIT responses upon infections with several viruses, including influenza A and B viruses, dengue virus and hepatitis C virus [111].

Upon activation, MAIT cells upregulate the expression of early activation markers including CD69 and CD107a, and secrete pro-inflammatory cytokines including IFN-γ, IL-17, TNF and GM-CSF [87, 97]. Notably, in response to different stimuli such as cytokines provided by other immune cells, MAIT cells can skew their cytokine production profiles, which lead to the definition of three MAIT cells subsets: MAIT-1, MAIT-17 and MAIT-1/MAIT-17 subsets, which are similar to Th1, Th17 and Th1/Th17 effector phenotypes in cytokine production and transcription factor expression [72, 112, 113]. With MAIT-1 phenotype, MAIT cells express a T-box transcription factor, T-bet, and can produce IFN-γ and TNF, which have been reported to either enhance innate phagocytosis or recruit other immune cells [114]. MAIT-17

cells, in contrast, express the transcription factor RORγt and preferentially produce IL-17, which, among other roles, helps in the recruitment of neutrophils [115]. Therefore, cytokine production is thought to be an important anti-microbial property of MAIT cells in either phenotype.

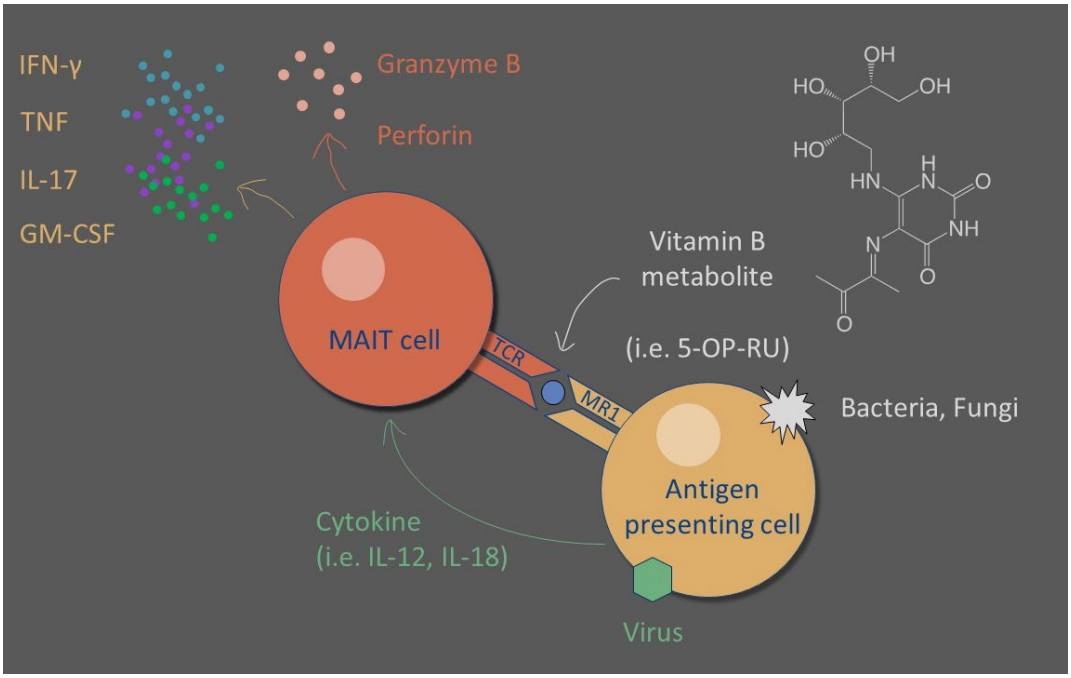


Figure 2 Activation of MAIT cells during infection.

Figure shows the activation of MAIT cells induced by antigen presenting cells in either a TCR-dependent (grey arrow) or TCR-independent (green arrow) manner. Bacterial and fungal infection can induce TCR-dependent activation when the microbe derived riboflavin metabolites (i.e. 5-OP-RU) conjugate with MR1 and are presented to MAIT cells via TCR binding. The skeletal formula of 5-OP-RU is shown. Virus and some bacteria/fungi trigger cytokine production in immune cells, and particular cytokines (i.e. IL-12 and IL-18) can activate MAIT cells in a TCR-independent manner. Activated MAIT cells can release pro-inflammatory cytokines including IFN-γ, TNF, IL-17 and GM-CSF. MAIT cells can also release cytotoxic molecules such as granzyme B and perforin.

Apart from cytokine production, another effector function of MAIT cells is cytotoxicity. Upon activation, MAIT cells can kill infected cells by releasing granzyme B and perforin, in a similar manner to cytotoxic T cells [72, 116]. Moreover, the expression of cytotoxic granzymes is well controlled in MAIT cells, as resting human MAIT cells mainly express granzyme A and K, while only stimulated MAIT cells are licensed to produce and release granzyme B [116]. Taken together, these properties have suggested that activated MAIT cells have potential protective roles in controlling microbial infection.

1.3 MAIT cell in bacterial infection diseases

Existing research recognizes the critical role played by MAIT cells against bacterial [75, 101, 106, 109, 110, 117-119] and viral infections [111, 120-124]. In a vast array of non-infectious diseases, including autoimmune diseases [78, 80], graft-versus-host disease after transplantation [81, 125], cancer [81, 126], obesity and type II diabetes [127], the involvement of MAIT cells has also been implicated. Herein, this section will focus on reviewing the MAIT cell responses in bacterial infection diseases.

It is noted that, in research of antibacterial properties of MAIT cells, current studies have been performed in either human samples or in mice, which share similarities but admittedly differ in many aspects such as percentage of MAIT cells and sensitivity to infections [73]. Therefore, human and murine MAIT cell studies are reviewed separately in this section.

1.3.1 Human studies

The exploration of MAIT cell antibacterial properties started from human studies. In the first set of published literature, human MAIT cells were found to be activated in an MR1-dependent manner when co-cultured with antigen presenting cells infected with bacteria, including *Escherichia. coli, Salmonella* Typhimurium*, Staphylococcus aureus, Mycobacterium spp., Lactobacillus acidophilus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae,* but surprisingly, not by *Listeria monocytogenes, Streptococcus group A* or *Enterococcus faecalis* [75, 109]. This conundrum was not resolved until the characterization of the MAIT cells stimulatory ligands by Kjer-Nilsen *et al.* and Corbett *et al.* [4, 5]. Notably, an *in vitro* activation assay was

developed for their purposes to detect bacteria derived ligands that specifically induce MAIT cell activation, by culturing Jurkat.MAIT cells (Jurkat T cells transduced with MAIT TCRs) with C1R.MR1 cells (Class I reduced C1R cells transduced with MR1) [4]. Utilizing this assay, it was conclusively shown that the particular bacteria (i.e. *E. faecalis*) that failed to induce MAIT activation was in consequence of the disability in generating MAIT cell ligands, as those bacteria do not possess a riboflavin pathway [5].

Thus far, the activation of human MAIT cells have been extensively studied upon infection of a wider range of bacteria. Clinically common pathogenic bacteria including *Streptococcus pneumoniae* [128], *Shigella flexneri* [117], *Helicobacter pylori* [118] and *Legionella longbeachae* [101] have been reported to stimulate MAIT cells after being engulfed by macrophages. In light of the ubiquitous expression of MR1 molecules, other types of cells, specifically dendritic cells [129, 130], monocytes [128, 131], B cells [132, 133] and epithelial cells [134], were also found to activate MAIT cells *in vitro* with the presence of either live or paraformaldehyde (PFA) fixed bacteria [75]. Moreover, the MAIT cell activation has been suggested not entirely MR1-dependent but could be induced by cytokines, mostly IL-12 and IL-18, provided by infected APCs. This has been supported by failed or incomplete blockage of MAIT cell activation during *S. pneumoniae* and *E. coli* infection with anti-MR1 antibodies, and the successful suppression of activation with anti-IL12/18 antibodies [117, 128, 135].

When investigating the mechanisms of MAIT cell protection, both cytokine production and direct killing of infected cells have been reported. IFN-γ production has been found in MAIT cells stimulated with *M. tuberculosis* [130], *S. pneumoniae* [129] and superantigens derived from *S. aureus* [135]. Human MAIT cells can also secrete other pro-inflammatory cytokines such as TNF and GM-CSF, which are important molecules that drives APC differentiation thus involved in mobilizing adaptive immunity [101]. In addition, MAIT cells are also capable of directly attack *E. coli* and *S. flexneri* infected epithelial cells by releasing cytotoxic molecules such as granzyme B [116, 117]. Interestingly, upon simultaneous stimulation of *E. coli,* circulating MAIT cells produced IFN-γ and granzyme B upon infection, while MAIT cells from female genital tract tend to generate more IL-17 and IL-22, suggesting functionally distinct phenotypes of MAIT cells are tendentiously distributed in human [97]. Taken together, these results suggest an important role is played by MAIT cells in controlling bacterial infection.

While most of research were performed *in vitro*, MAIT cells have also been investigated *ex vivo* with human patients by observational and correlative analysis. Clinical studies have suggested that patients of infectious diseases have fewer circulating MAIT cells compared to healthy individuals, as assessed by either absolute number or proportion. So far, this particular observation has been reported from patients suffering infections of *M. tuberculosis* [75, 109, 136], *H. pylori* [118], *P. aeruginosa* [99], *Vibrio cholerae* [122] as well as people receiving a *Shigella* vaccine [117], or a *Salmonella* vaccine [137]. An explanation is that circulating MAIT cells might migrate to the site of infection, where they enhance local immunity and control the infection [75]. This has been firstly supported by a series of findings, in which MAIT cells were detected in the urine of patients with urinary tract infection [90], in the lung of pulmonary tuberculosis patients and in the ascites fluid of disseminated tuberculosis patients [75, 136]. Secondly, Grimaldi *et al.* showed that critically ill patients with sepsis had a remarkably reduced level of circulating MAIT cells, compared to non-septic critically ill patients [138]. It is therefore suggested that the decrease of circulating MAIT cells were induced by the developing infections but not by pathologically destruction of organs due to other critical diseases. Thirdly, circulating MAIT cells were found activated after infection of *V. cholerae* or *Shigella* with upregulation of activation markers such as CD38 and HLA-DR in MAIT cells [117, 122], suggesting they could play an antimicrobial role once recruited to the site of infection. Overall, these results support the hypothesis that MAIT cells are activated and potentially accumulated in the infection sites, thus potentially protective during bacterial infections in human.

1.3.2 Mouse studies

The use of mouse as models of human immunology studies has allowed physiological investigations of MAIT cells *in vivo*, and many of which could not be conducted in human. However, due to the scarcity of MAIT cell in mice in contrast to that of humans [1, 72, 82], previous studies have sought for improvement of MAIT cell abundance to facilitate research of MAIT cells. This has been achieved by overexpressing MAIT iTCRα genes (Vα19*i*) and/or TCRβ gene (Vβ6/8) in mice, and the resultant TCR-transgenic (Tg) mice harboured significantly higher numbers of MAIT cell [75, 84, 139]. Moreover, knockout of the endogenous TCRα gene ($C\alpha^{-/-}$) further elevated the MAIT cell percentage in the Vα19*i*Tg mice [84, 139]. Also, higher MAIT iTCR expression was found in the wild derived inbred CAST mice, and by

introducing MAIT^{CAST} loci to C57BL/5 background, Cui *et al.* generated C57BL/6-MAIT^{CAST} mice which had more MAIT cells. Utilizing either wildtype or transgenic mice, MAIT cell responses were studied *in vivo* during bacterial infections in the lung, urinary tract and peritoneal cavity (Table 2).

Table 2 Established mouse models studying MAIT cell response upon bacterial infection.

Site of infection	Microorganisms	Mouse strain	Reference
Urinary tract	*Escherichia coli*	C57BL/6-MAIT^{CAST}	(Cui *et al.*, 2015)
Stomach	*Helicobacter pylori*	C57BL/6 Vα19*i*Tg*Cα*$^{-/-}$	(D'Souza *et al.* 2018)
Peritoneal cavity	*Klebsiella pneumoniae*	C57BL/6	(Georgel *et al.*, 2011)
	Escherichia coli	Vα19*i*Tg	(Bourhis *et al.*, 2010)
Lung	*Salmonella* Typhimurium	C57BL/6	(Chen *et al.*, 2017)
	Legionella longbeachae	C57BL/6	(Wang *et al.*, 2018)
	Francisella tularensis	C57BL/6	(Meierovics *et al.*, 2013)
	Mycobacterium bovis BCG	C57BL/6, Vα19*i*Tg*Cα*$^{-/-}$	(Sakala *et al.*, 2015)

Among the published studies, most attention has focused on pulmonary infections, and MAIT cells have been reported essential for full control of *L. longbeachae* [101], *Francisella tularensis* [140] and *M. bovis* BCG [141] *in vivo*. This has been concluded by comparing the microbial burden in the lung between wild type C57BL/6 (*Mr1*$^{+/+}$) mice and *Mr1*$^{-/-}$ (MAIT cell deficient) control mice, and significantly higher burdens were observed in *Mr1*$^{-/-}$ mice in each case [101, 140, 141]. Notably, the mechanisms of MAIT cell protection have also been investigated in a few studies. Firstly, it has been found that MAIT cells number and percentage significantly increased in the lung after inoculation of all the three bacteria as well as *Salmonella* Typhimurium, an unnatural causative organism of pulmonary infection [95]. These results, which are in correlation with that of human patients with local infections, suggest that the MAIT cells preferential amassed in the lung and potentially contribute to the clearance of bacteria. Secondly, cytokine production analysis showed that the accumulated MAIT

cells were producing pro-inflammatory cytokines, including IFN-γ, IL-17 and GM-CSF, during the early phase of infection. During pulmonary infection of *F. tularensis* LVS, impaired cytokine production as well as delayed recruitment of conventional T cells were observed in *Mr1*$^{-/-}$ mice [140]. It was further demonstrated in this model that MAIT-source of GM-CSF was indispensable for DC accumulation and maturation, thus indirectly influenced adaptive immune responses [142]. Similarly, MAIT cells produced an array of cytokines upon *L. longbeachae* infection, while the protective role of MAIT cells relied on IFN-γ and GM-CSF but not IL-17 [101]. Production of IL-17 and IFN-γ have been observed in lung MAIT cells from *S. pneumoniae* infected mice, although no protection mediated by MAIT cells has been found [129].

A few studies have also investigated MAIT cells upon infection of organs other than the lungs. Comparison studies show that MAIT cells are potentially involved in controlling the growth of bacteria in the urinary tract and peritoneal cavity in mice. Utilizing a MAIT-rich congenic strain of mouse (C57BL/6-MAIT^{CAST}), Cui *et al.* demonstrated MAIT cells displayed protective activity upon urinary tract infection of *E. coli* [90]. When assessing infections within the peritoneal cavity, Le Bourhis *et al.* found that Vα19iTg *Mr1*$^{-/-}$ mice had higher bacterial burdens after intraperitoneal infection of *E. coli* compared to the *Mr1*$^{+/+}$ counterparts [75]. Conversely, Georgel *et al*. reported that no significant difference of peritoneal cavity bacterial loads has been found between C57BL/6 and *Mr1*$^{-/-}$ mice after inoculation of luminescent *E. coli*, *Shigella dysenteriae* or *Yersinia enterocolitica* [143]. In the same study, a higher mortality as well as a delayed bacterial clearing process were observed in *Mr1*$^{-/-}$ mice post *K. pneumoniae* infection, compared to their wildtype counterparts [143].

Apart from *in vivo* studies, murine MAIT cells have also been studied *in vitro,* which has been beneficial for linking human and murine MAIT cells for a more comprehensive view. In a wide-ranging examination of MAIT cell responses, Le Bourhis *et al.* showed marked activation of purified murine MAIT cells in response to PFA-fixed various bacteria such as *K. pneumoniae* and *S. aureus,* but not *E. faecalis* or group A *Streptococci* [75]. Moreover, MAIT cells were activated in a TCR-dependent manner, which strongly correlated with results obtained with human MAIT cells [75]. *In vitro* studies also allow further characterization of MAIT cell protection mechanisms against a certain bacterium. Specifically, it has been reported that MAIT cells controlled intracellular growth of *M. bovis* BCG *in vitro* by producing cytotoxic nitrite (NO_2^-), and this process strongly depended upon IL-12, which was provided by the infected bone marrow derived macrophages [114]. A similar observation was

reported during *F. tularensis* infection as well, while the mechanisms remained unknown [140].

Despite their innate-like properties, MAIT cells were suggested to be capable of forming memory cells [84, 144]. Memory MAIT cells were indicated in a vaccination-then-challenge study with *L. longbeachae* infection [101], in which a MAIT cell based vaccination with TLR agonists plus synthetic ligand 5-OP-RU provided a better control of bacteria in mice.

1.4 Research aims

MAIT cells have been characterized by their innate-like anti-microbial functions. It has been reported that MAIT cells play important roles in several infectious diseases, but the mechanism and properties of MAIT cells are still not fully elucidated, particularly in the context of a complex immune response.

To date, few mouse models have been established for studying MAIT cell responses during bacterial infections *in vivo*, expecially infections that happened outside the lung. In particular, it remains irreproducible whether MAIT cells are protective upon peritoneal cavity infection , or peritonitis, by *K. pneumoniae* [143]. While human observatory and correlative analysis suggestted that MAIT cells amassed in the ascites of patients suffering from infection [75], conflicting results were reported from the scant animal studies [75, 140]. Given that peritonitis is a clinically-common and life-threatening disease [145, 146], it is of importance to further investigate the protection afforded by MAIT cells against this particular clinical complication. Therefore, this project seeks to establish models that can be used to examine the role of MAIT cells in animal infection models with three most frequently found bacteria in peritonitis patients in clinic.

In addition, while extensive studies have demonstrated that MAIT cells play a pivotal role in controlling microbes, either by killing infected cells directly or secreting cytokines, the initiation of MAIT cell responses during infection has been less explained. Utilizing cell lines, sorted MAIT cells or human peripheral blood mononuclear cells (PBMC), several studies have reported that bacterially-infected macrophages and dendritic cells were able to activate co-cultured MAIT cells *in vitro* [75, 118, 147]. However, given the complexity of immune response *in vivo* as well as the wide expression of MR1 in mammalian cells, it has not been elucidated which

types of cell preferentially act as APCs for MAIT cel activation during bacterial infection. Hence, this study is also aiming to reveal those preferred APCs for MAIT cell activation upon *in vivo* bacterial infection and due to the limitation of current methods, this study also seeks to establish a suitable cellular system reflecting *in vivo* responses.

To recapitulate, this dissertation aims to develop new models which would benefit MAIT cell studies and further characterise the MAIT cell responses during bacterial peritonitis and pulmonary infection. The specific aims of this project are to:

1. Establish *in vivo* peritonitis models for MAIT cell studies with the most frequently found bacteria clinically (Chapter 3).
2. Explore the protective function and immune response of MAIT cells against bacterial peritonitis (Chapter 3).
3. Establish an *ex vivo* cellular system for defining the antigen presenting cell subsets for MAIT cell activation (Chapter 4).
4. Define the antigen presenting cell subset for MAIT cell activation during *Francisella tularensis* LVS pulmonary infection (Chapter 4)

Chapter 2: Material and Methods

2.1 Material: reagents and buffers

Table 3 Reagents and buffers.

Name	Components	Amount	Manufacture
Blank calibration particles (6.0-6.4 um)	N/A		Sphero BD Bioscience
Brain Heart Infusion (BHI) broth/agar plate	Brain heart infusion (Oxoid, CM1135)	37 g/L	Media Preparation Unit
	Agar	10 g/L	
Cysteine Heart Agar (CHA)	Ampicillin	10 mg/L	Media Preparation Unit
	Colistin	7.5 mg/L	
	Sheep blood	9%	
	Trimethoprim	4 mg/L	
	Agar	10 g/L	
Collagenase digestion solution	DNAse I	2.5 mg	Roche Diagnostics GmbH
	FCS	10 ml	HyClone Thermo Scientific
	RPMI	490 ml	Media Preparation Unit
	Type III collagenase	1.5 g	Worthington Biochemical Corporation
Dimethyl sulfoxide (DMSO)			Sigma-Aldrich
Ethanol			Chem-Supply
Fluorescence-activated cell sorting (FACS) fix	D-glucose	2%	Chem-Supply
	Formaldehyde	1%	Univar
	PBS		Media Preparation Unit
FACS wash	FCS	1% or 2%	HyClone Thermo Scientific
	PBS		Media Preparation Unit
Fetal calf serum (FCS)			HyClone Thermo Scientific
Fixation/Permeabilization buffer, working solution	Kit component		BD Bioscience
Foxp3/Transcription factor Fixation/Permeabilization buffer, working solution	Kit component		eBioscience

Glycerol			Chem-Supply
Golgi plug			BD Bioscience
Ionomycin			Sigma-Aldrich
Luria broth/agar plate	Tryptone	10 g/L	Media Preparation Unit
	Yeast extract	5 g/L	
	NaCl	10 g/L	
	Agar	10 g/L	
moMR1 monoclonal antibody	Clone: 8F2.F9 (IgG1) Isotype control: 3E12		Gift from Ted Hansen [109]
Percoll			GE Healthcare
Phorbol myristate acetate (PMA)			Sigma-Aldrich
Phosphate-buffered saline (PBS)	NaCl, KCl Na_2HPO_4, KH_2PO_4		Media Preparation Unit
RF-10 media	FCS	60 ml	HyClone Thermo Scientific
	RPMI 1640	500 ml	Gibco Life Technologies
	SC	30 ml	Made in house
Roswell Park Memorial Institute (RPMI) 1640 media			Gibco Life Technologies
Streptomycin sulfate salt		767 I.U./mg	Sigma-Aldrich
Supplementary components (SC)	β-mercaptoethanol	35 µl	Sigma-Aldrich
	HEPES	11.9 g	Gibco Life Technologies
	L-glutamine	3 g	Gibco Life Technologies
	Non-essential amino acid solution	100 ml	Gibco Life Technologies
	Penicillin-streptomycin solution	80 ml	Gibco Life Technologies
Tris-borate-EDTA (TBE) buffer	Boric acid	17.8 g	Chem-Supply
	EDTA (0.5M, pH8.0)	20 ml	Sigma-Aldrich
	Milli-Q water	10 L	ELGA labwater
	Tris	54 g	Ajax Finechem
Tris-buffered ammonium chloride (TAC) red blood cell lysis solution	HCl (2M)	to pH7.2	
	NH_4Cl	14.94 g	Sigma-Aldrich
	Milli-Q water	1900 ml	ELGA Labwater
	Tris (1M, pH8.0)	34 ml	Ajax Finechem
Trypan blue			Sigma-Aldrich
UltraComp eBeads Compensation Beads			Invitrogen

2.2 Methods

2.2.1 Ethics

Mouse experiments in this study were approved by the University of Melbourne, Biochemistry & Molecular Biology, Dental Science, Medicine, Microbiology & Immunology, and Surgery Animal Ethics Committee under two approvals.

Ethics ID: 1513661 (Sep 2015 – Dec 2018) Mucosal Associated-Invariant T (MAIT) cell immunity in bacterial infection, and Ethics ID: 1814616 (Dec 2018 -) Investigation of Mucosal Associated-Invariant T (MAIT) cell immunity in mice

2.2.2 Bacteria and growth conditions

Bacteria stocks were stored at -80°C in broth with 50% glycerol. Growth of single colonies was achieved by streaking glycerol stock to an agar plate with appropriate antibiotics. Streaking was accomplished by dragging 10 µl stock with inoculation loop in a continuous zigzag. Inoculation loop was sterilized by passing through a flame beforehand and between streaks. Colonies would be visible 1-4 days post inoculation, depending on the growth rate of individual bacteria.

A single colony was picked from streaking plate and inoculated to 10 ml broth. Bacteria were cultured overnight at 37°C with or without shaking and re-inoculated with fresh medium on the day of infection. The concentration of bacteria was estimated by optical density (OD) values at 600 nm at log phase, and bacteria were serially diluted to meet experiment intention.

Sample of infection inoculum was spread on agar plates to verify the actual concentration of bacterial inoculum by colony forming unit (CFU) counts. By serial dilution, an estimation of 100 bacteria contained in 100 µl was spread on plates and colonies enumerated after incubation at 37°C. Colonies were counted and the morphology of colony was confirmed to rule out contamination. The information was recorded and accurate estimation of concentration of bacteria was achieved after repeated practice and modification of the estimation CFU/$OD_{600\ nm}$ constant.

Table 4 Strains of bacteria that were used in this study with culture conditions.

Bacterium	Strain	Broth	Plate	Antibiotics	Condition
Francisella tularensis	LVS	brain heart infusion broth (BHI)	Cysteine heart agar (CHA)	A mix of antibiotics*	200 rpm**
Legionella longbeachae	NSW150 [148]	buffered yeast extract (BYE)	buffered charcoal yeast extract agar (BCYE)	50 μg/ml streptomycin	200 rpm
Klebsiella pneumoniae	B5055 7825	Luria Bertani broth (LB)	Luria Bertani agar (LB)	N/A	200 rpm
Staphylococcus aureus	BPH 2986	brain heart infusion broth (BHI)	brain heart infusion agar (BHI)	***	200 rpm
	BPH 2819				
	BPH 2760				
	BPH 2900				
	BPH 2947				
Escherichia coli	ST131	Luria Bertani broth (LB)	Luria Bertani agar (LB)	50 μg/ml streptomycin	still

*, 10 μg/ml ampicillin, 7.5 μg/ml colistin and 4 μg/ml trimethoprim.

**, Rpm, revolutions per minute.

***, Resistant to clinically common antibiotics:

1. BPH 2986: oxacillin, erythromycin, fosfomycin, aminoglycoside;
2. BPH 2819: fosfomycin
3. BPH 2760: penicillin
4. BPH 2900: oxacillin
5. BPH 2947: oxacillin, spectinomycin, erythromycin, tetracycline, fosfomycin, aminoglycoside

2.2.3 Bacterial supernatant and lysate preparation

10 ml of bacterial culture broth at a desired growth phase was centrifuged at 3400 x *g* (x *g*, times gravity) for 10 minutes at 4°C. The supernatant was sterilized by passing through 0.22 μm syringe filter. Bacterial pellets were washed and re-suspended in 1 ml ice-cold PBS. Bacteria were lysed by sonication with probe to achieve complete membrane rupture, and bacterial lysate was filter-sterilized with 0.22 μm syringe filter.

Aliquots of supernatant and lysate were stored at -80°C for future use.

2.2.4 Bacterial infection to mice

Bacteria were titrated in PBS to a planned concentration before delivered to mice. C57BL/6 mice or gene knockout mice aged 6-12 weeks were infected by different routes, including intravenous (i.v.), intraperitoneal (i.p.) and intranasal (i.n.). I.v. and i.p. infections were achieved by injecting 200 μl of prepared bacterial inoculum with an insulin syringe. When infecting mice by the i.n route, mice were first anesthetized with an isoflurane device, and 50 μl inoculum containing the desired number of bacteria was instilled drop by drop to the nostril with a 200 μl pipette.

Mice were monitored accordingly after infection.

2.2.5 Assessment of bacterial load by CFU analysis

Mice were euthanized with CO_2 asphyxiation, and organs were perfused with 10 ml cold RPMI through the right ventricle. The lung, liver, spleen and kidney were collected and held in 3 ml RPMI. A peritoneal wash was performed by injecting 10ml cold sterile PBS i.p., and resulting fluid was collected with a syringe after 2-minute abdomen massage.

Organs were homogenized with a homogenizer at 21,000 rpm for 20 seconds. The bacterial burden was enumerated by CFU assay after spreading 100 μl serial dilutions of organ homogenates and peritoneal lavage fluids on agar plates. Plates were incubated at 37°C and bacterial load was expressed as the number of CFU per organ or per peritoneal cavity.

2.2.6 Preparation of single cell suspension from organs

Organs were harvested as mentioned. Lung and kidney were cut into fine pieces with surgical scalpel blades and placed in digestion solution containing collagenase. Collagenase digestion was accomplished by incubating the organ pieces at 37°C with continuous shaking at 120-180 rpm for 90 minutes (lung) or 45 minutes (kidney). Liver, spleen and the digested lung and kidney were pushed through 70 μm strainers to remove tissue clumps. Red blood cells were lysed in Tris-buffered ammonium chloride (TAC) for 5 minutes at 37°C water bath. Cells were washed twice with FACS wash to remove TAC solution.

For liver and kidney, Percoll fractionation was performed to deplete epithelial cells. Cells were then washed twice with FACS wash and were filtered through 100 μm strainers to 5 ml FACS tubes.

2.2.7 Fractionation of lymphocytes using Percoll

Percoll working solutions (36 % and 70%) were prepared beforehand by diluting stock 100% Percoll (100%, density 1.13 g/mL) in PBS (Table 5).

Table 5 Formula of preparing Percoll working solution.

	Percoll stock	1x PBS	10x PBS	Total volume
36% Percoll	54 ml	90 ml	6 ml	150 ml
70% Percoll	63 ml	20 ml	7 ml	90 ml

Cells were spun down in 10 ml centrifuge tube. Cell pellet was re-suspended in 5 ml 36% Percoll and gently underlay the cell suspension with 3 ml of 70% Percoll solution. Cells were centrifuged at 900 × *g* for 20 minutes at room temperature with brake OFF. After centrifugation, the layer of epithelial cells at the topside of 36% Percoll was discarded by aspiration. Lymphocytes and other immune cells formed a pale layer between 36% and 70% Percoll solutions, which were collected into a separate tube.

2.2.8 Surface and intracellular staining

2.2.8.1 Surface staining

Cells were firstly spun down for surface staining. Nonspecific antibody binding was blocked with 20 µl blocking solution, which contains Fc receptor antibody (clone 2.4G2) and MR1-6-FP tetramer, for 15 minutes at room temperature. MR1-6-FP tetramer was prepared by tetramerizing MR1-6-FP monomers with SA (molar ratio at 4:1) as described previously [5, 73]. Cells were then stained in 40 µl mixture of antibodies/MR1-5-OP-RU tetramers diluted in PBS for 30 minutes and were vortexed every 15 minutes. Cells were washed twice with FACS wash and re-suspended in FACS fix.

The standard panels of antibodies for staining MAIT cells and APCs were listed in Table 6 and Table 7 respectively.

Table 6 Staining panel of murine MAIT cells.

Antibody	Clone	Fluorochrome	Manufacture	Concentration (mg/ml)	Dilution
7AAD			BD Bioscience		1:500
CD45.2	104	FITC	BD Bioscience	0.5	1:200
CD19	1D3	PerCP-Cy5.5	BD Bioscience	0.2	1:200
CD69	H1.2F3	PE	Invitrogen	0.2	1:200
TCRβ	H57-597	APC	BD Bioscience	0.2	1:200
MR1 5-OP-RU tetramer*	N/A	BV421	Made in-house	0.185	1:200
CD4	GK1.5	APC-Cy7	BD Bioscience	0.2	1:200
CD8a	53-6.7	PE	BD Bioscience	0.2	1:500

*MR1-5-OP-RU tetramers were prepared by tetramerizing MR1-5-OP-RU monomers with SA-BV421 (molar ratio at 4:1) as described previously [73] and specifically stains MAIT cells.

Table 7 Staining panel of APCs in the mouse lung.

Antibody	Clone	Fluorochrome	Manufacture	Concentration (mg/ml)	Dilution
7AAD			BD Bioscience		1:500
CD45	30-F11	PerCP	eBioscience	0.2	1:100
SiglecF	E50-2440	PE-Cf594	BD Bioscience	0.2	1:1000
CD11b	M1/70	APC-Cy7	BD Bioscience	0.2	1:100
CD11c	N418	FITC	eBioscience	0.2	1:200
CD103	2E7	PE	BioLegend	0.2	1:200
Ly6G	1A8	PE-Cy7	BD Bioscience	0.2	1:200
MHCII	M5/114.15.2	BV421	BioLegend	0.05	1:100
CD64	X54-5/7.1	BV711	BioLegend	0.2	1:200

2.2.8.2 Intracellular cytokine staining (ICS)

Cells were first stimulated with 20 ng/ml PMA and 1 μg/ml ionomycin *ex vivo* for 3 hours (37°C, 5% CO_2) before staining. To gate out the dead cells, cells were first washed with PBS once and stained with 1 ml of diluted FVD on ice for 30 minutes. After washed twice with FACS wash, cells were stained for surface markers as previously described, then ICS was performed by following the manufacturer's instruction. The standard panels of antibodies for staining MAIT cells were listed in Table 8.

Cytokine staining in this study was based on instruction manuals provided in the Fixation/Permeabilization Kit by BD Bioscience.

2.2.8.3 Transcription factor (TF) staining

TF staining was carried out similarly as ICS that is described above but without *ex vivo* stimulation and use a different reagent kit, which is optimized for intranuclear protein staining. The standard panels of antibodies for staining MAIT cells were listed in Table 8.

Transcription factor staining in this study was based on instruction manuals provided in the Foxp3/Transcription Factor Fixation/Permeabilization Kit by eBioscience.

Table 8 Staining panel of cytokines and transcription factors of mouse cells.

Antibody	Clone	Fluorochrome	Manufacture	Concentration (mg/ml)	Dilution
FVD		eFluor 780	eBioscience		1:1000
CD45.2	104	FITC	BD Bioscience	0.5	1:200
CD19	1D3	PerCP-Cy5.5	BD Bioscience	0.2	1:200
TCRβ	H57-597	APC/PE *	BD Bioscience	0.2	1:200
MR1 5-OP-RU Tetramer	N/A	BV421	Made in house	0.185	1:200
CD44	IM7	BUV737	BD Bioscience	0.2	1:200
IFN-γ	XMG1.2	PE-Cy7	BD Bioscience	0.2	1:400
IL-17	TC11-18H10	PE	BD Bioscience	0.2	1:200
RORγt	B2D	APC	eBioscience	0.2	1:200
T-bet	4B10	PE-Cy7	eBioscience	0.2	1:200

*, TCRβ-APC was used in cytokine staining, TCRβ-PE was used in transcription factor staining.

2.2.9 Flow cytometry and cell sorting

Flow cytometry data acquisition was performed with BD Fortessa flow cytometer and data analyzed by Flowjo v10 (Treestar).

To estimate the absolute number of cell populations, a certain number of beads (Blank Calibration Particles) were added to the prepared sample after estimation of bead concentration by bead counting with a Hemocytometer. The cell number was therefore deduced from the ratio of beads number/cells number showed in the FACS plot.

Sorting of uninfected cells was performed on BD Aria III cell sorter, and infected cells were sorted with MoFlo Astrios EQ Sorter. Purity was typically over 90%. Sorted cells were then used for determining intracellular bacterial load, *ex vivo* culture and activation assay.

Mouse MAIT cells were gated by 7AAD^{-}, CD45.2^{+}, CD19^{-}, TCRβ^{+}, MR1 5-OP-RU tetramer^{+} population without auto-fluoresce (Figure 3). Lung APC subsets were gated as shown in Chapter 4 (Figure 16).

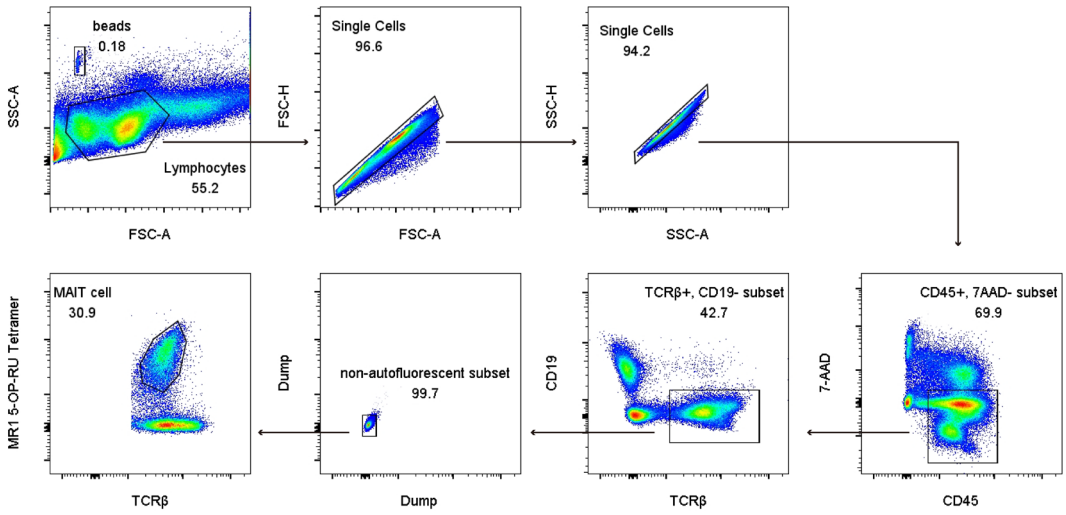


Figure 3 Gating strategy to identify murine MAIT cells.

Mouse MAIT cells were gated by 7AAD^{-}, CD45.2^{+}, CD19^{-}, TCRβ^{+}, MR1 5-OP-RU tetramer^{+} population after exclusion of auto-fluorescence. Beads and lymphocytes were gated by forward versus side scatter, and single cells discriminated from scatter area versus height. 7-AAD^{+} or FVD^{+} dead cells were excluded from the CD45^{+} population, and CD19^{+} B cells were excluded from TCRβ^{+} population. Autofluorescent cells were excluded by displaying cells with Violet 525/50 versus Violet 660/20, which flanking BV421.

2.2.10 Ex vivo activation assay

To prepare cells for *ex vivo* activation assay, mice were killed, and organs were perfused by injecting 10 ml of cold RPMI through the left ventricle before lungs were harvested. This was performed to purge away blood cells and enrich tissue interstitial lymphocytes. Single cell suspensions were prepared, and cells were stained for flow cytometry sorting as previously described. The panels of antibodies for staining cells were listed in Table 6 and Table 7. To improve MAIT cell yield and purity for cell sorting, immune cells were further enriched by Percoll fractionation when sorting MAIT cells from lung.

MAIT cells were sorted with BD Aria III cell sorter, while APCs from acutely infected mice were sorted with MoFlo Astrios EQ sorter in a biosafety cabinet. Cells were collected in ice-cold FCS during sorting and were re-suspended in warmed RF-10 before cultured in 96-well U bottom plate *ex vivo*. Each well contained 10^4 MAIT cells and 10^4 APCs in 180 µl. When adding controlling ligand or antibodies to wells, stock was diluted in RF-10 first and add 20 µl to each well, otherwise add 20 µl of RF-10.

Cells were incubated at 37°C, 5% CO_2 incubator for 5 hours to allow cell interaction and MAIT cell activation. Transfer cells to V bottom well plates and stain cell as described previously. The panel of staining *ex vivo* cultured cells is shown in Table 9. Stained cells were then analyzed by flow cytometry in high-throughput sampler (HTS) mode.

Table 9 Staining panel of *ex vivo* activation assay.

Antibody	Clone	Fluorochrome	Manufacture	Concentration (mg/ml)	Dilution
CD69	H1.2F3	PE	Invitrogen	0.2	1:200
TCRβ	H57-597	PAC	BD Bioscience	0.2	1:200
MR1 5-OP-RU Tetramer	N/A	BV421	Made in-house	0.185	1:200

2.2.11 Cell lines and activation assay

C1R.MR1 cell line is an Epstein - Barr Virus transformed human lymphoblastoid B cell line with overexpression of human MR1. Jurkat.MAIT cells are immortalized human T lymphocytes transduced with human MAIT TCR [149]. Human cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and incubated in 37 °C, 5% CO_2.

Activation assay was performed with 96-well U-bottom plate with 200 µl volume each well. Typically, one well contains 90 µl of C1R.MR1 cells (10^5), 90 µl of Jurkat.MAIT cells (10^5) and 20 µl of reagents, i.e. MAIT cell ligand 5-OP-RU or 6-FP, which add up to 200 µl. Synthetic 5-OP-RU and 6-FP was made as previously described [5]. After overnight incubation, cells were transferred to a 96-well V-bottom plate and stained for FACS. The panel of staining is presented in Table 10. Jurkat MAIT cells were gated as $7AAD^-$, $CD3^+$ population.

Table 10 Staining panel of *in vitro* activation assay.

Antibody	Clone	Fluorochrome	Manufacture	Concentration (mg/ml)	Dilution
7AAD			BD Bioscience		1:500
CD3	UCHT1	APC	BD Bioscience	0.2	1:50
CD69	FN50	PE	BD Bioscience	0.2	1:50

2.2.12 Statistical Analyses

All statistical tests were performed using Prism software (version 6, GraphPad Software, Inc., La Jolla, California, USA). Comparisons between groups were conducted using Student's t-test, unless otherwise stated. Survival curves were compared using log-rank tests. Significance was defined with a probability (*P*) value depicted by asterisks: *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001.

Chapter 3: Establishing peritonitis models for assaying MAIT cell roles using representatives of those most frequently found bacteria clinically.

3.1 Introduction

Extensive research has suggested that a pivotal role is played by MAIT cells in the immune response to bacterial infection [75, 95, 99-101, 106, 114, 118, 135, 140, 150, 151]. However, human MAIT cell research methods to date have been restricted to *in vitro* cell line studies [4, 101, 149] and analysis of MAIT cells from PBMC and/or tissues samples [99-101, 106, 118, 135, 150, 151]. Therefore, the antibacterial nature of MAIT cells *in vivo* is less clear.

To explore the properties of MAIT cells in clinical bacterial infection *in vivo*, a reliable animal model is required. A few mouse models have been developed, as reviewed in Chapter 2 [90, 110, 118, 140, 143], and MAIT cells have shown protective roles in controlling infection of several bacteria: *F. tularensis* and *L. longbeachae* in the lung [101, 140], *E. coli* in the urinary tract [90] as well as *K. pneumoniae* in the peritoneal cavity [143]. However, current studies are still limited in number and incomprehensive. Therefore, this study aims at developing clinical relevant infection models in mice in order to further investigate the antibacterial properties of MAIT cells.

Specifically, peritoneal infection (bacterial peritonitis) was chosen as the main interest due to its frequent occurrence and high mortality in clinic. There are two types of bacterial pertionitis clinically, both of which can be life-threatening [152]. Spontaneous bacterial peritonitis (SBP) is a common but devastating complications of advanced liver cirrhosis, and is responsible for 30% to 50% of the mortality in hospitalized patients [153, 154]. Secondary bacterial peritonitis, although less frequent, has a higher mortality of 50%-80% [155]. It is noted that three bacteria, *K. pneumoniae*, *S. aureus* and *E. coli*, account for over 50% of the SBP cases and 80% of secondary peritonitis cases clinically [146, 156] (Table 11). Moreover, all three bacteria bear an intact riboflavin synthesis pathway [107], suggesting all are capable of activating MAIT cells. It was therefore plausible to optimize intraperitoneal infection models with the three most frequently found bacteria clinically (*K. pneumoniae*, *S. aureus* and *E. coli*) for MAIT cell assessment in this study.

Table 11 Causative microorganisms of spontaneous bacterial peritonitis and secondary peritonitis

Microorganisms	SBP%	Secondary peritonitis (%)
Escherichia coli	37/35.8	20
Klebsiella pneumoniae	17/15.5	7
Pneumococcus	12	0
Viridans Streptococci	9/10.4	0
Staphylococcus aureus	0	11
Polymicrobial	1	53

SBP, spontaneous bacterial peritonitis.

Reproduced from Sleisenger's & Fordtran's gastrointestinal and liver disease, 7th ed [146].

In this study, mouse peritonitis models were established with clinical strains of *K. pneumoniae*, *S. aureus* and *E. coli*. Two strains of *K. pneumoniae* B5055and 7825 are clinically isolated and mouse-adapted (personal communication, R. Strugnell) [157]. Five clinical isolates of *S. aureus* (BPH 2986, 2819, 2760, 2900 and 2947) and *E. coli* sequence type 131 (ST131), a pandemic strain causing extraintestinal infection [158], were also used for this study. MAIT cell responses when stimulated by the three bacteria were assessed in a step-by-step manner. Firstly, utilizing a well-established *in vitro* cellular MAIT activation assay [4, 5], it was first examined if the three bacteria produced MAIT cell antigens when growing *in vitro*. Secondly, *in vivo* peritonitis models of the three bacteria were trialed in C57BL/6 mice, and MAIT cell properties were subsequently investigated in each particular scenario of infection.

3.2 Results

3.2.1 Growth kinetics of Klebsiella pneumoniae, Staphylococcus aureus and Escherichia coli.

MAIT activating ligand 5-OP-RU is directly derived from the intermediate metabolite 5-A-RU from the vitamin B2 (riboflavin) synthesis pathway, which is possessed by most bacteria but not mammals. The presence of 5-OP-RU thus serves as a signature of bacterial infections. Although 5-OP-RU production has not previously been described, the production of the end product (riboflavin) has been extensively studied and strongly suggested its link with microbial growth rates [159, 160]. Therefore, it was presumed that the bacterial growth rates are closely related with the production of 5-OP-RU and thus correlate with MAIT cell activation. To test this hypothesis, growth kinetics of *K. pneumoniae*, *S. aureus* and *E. coli* were first determined. This information would also help to optimise the bacterial inoculum preparation for *in vivo* infections. Individual and comparative growth kinetics are depicted in Figure 4.

As shown in Figure 4, the three bacteria display similar but distinct growth kinetics. For *E. coli*, bacteria reached log phase (exponential phase) by 1 hour and plateaued at about 3 hours post re-inoculation. All strains of *S. aureus* had log phase growth between 1-3 hours post re-inoculation. Both *K. pneumoniae* strains had less typical growth kinetics, but the bacterial growth slowed down from 7 hours post re-inoculation. Therefore, it was decided to sample bacterial supernatant at the most representative time points for future MAIT cell activation assays:

1. 0 hr.: culture start, with least number of bacteria;
2. 1.5 or 2 hrs: early log phase, with rapidly growing bacteria;
3. 3 or 4 hrs: late log phase, with more rapidly growing bacteria than the 1.5-2 hrs time point;
4. 12 hrs: stationary phase, with slowly growing or static bacteria.

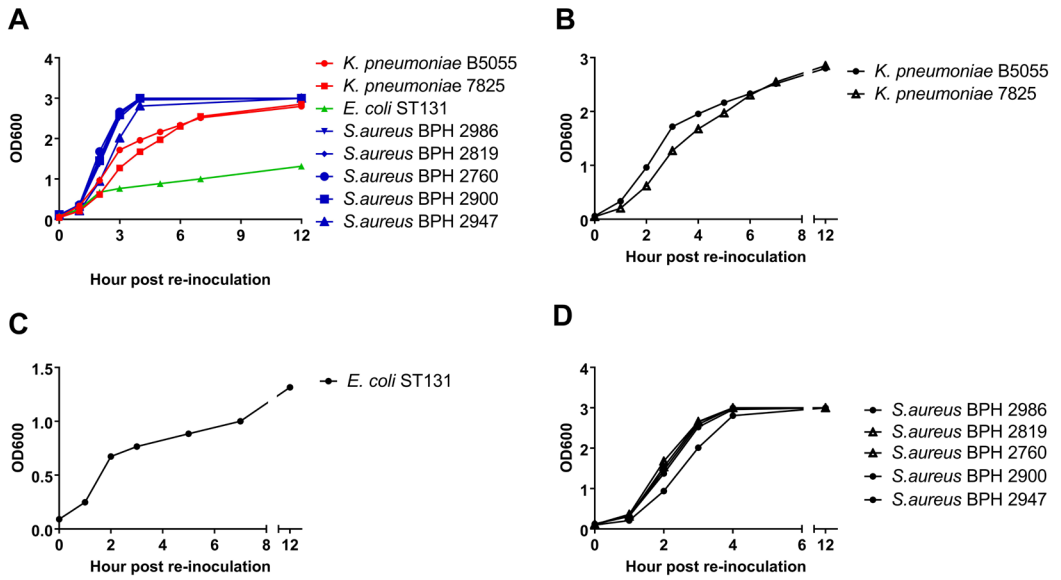


Figure 4 Growth kinetics of *K. pneumoniae*, *E. coli* and *S. aureus*.

A single colony from agar plates of individual bacteria was inoculated to broth with appropriate antibiotics and cultured overnight at 37ºC with shaking (for *K. pneumoniae* and *S. aureus*) or without shaking (for *E. coli*). 200 µl overnight culture was re-inoculated to fresh pre-warmed broth, and bacterial growth (broth turbidity) was monitored by measuring O.D. at 600nm (OD_{600nm}) at indicated time points (hours) post inoculation. A) Comparison of growth curves of *K. pneumoniae* (red), *E. coli* (green) and *S. aureus* (blue). Y-axis shows the OD_{600nm} value. X-axis shows hours post re-inoculation. Kinetics of individual bacteria are shown in B) two strains of *K. pneumoniae*; C) *E. coli* (ST131) and D) five strains of *S. aureus*. Experiment was performed once.

3.2.2 K. pneumoniae, S. aureus and E.coli produce antigens that activate a MAIT reporter cell line.

In order to probe if the clinical bacterial isolates can activate MAIT cells, an *in vitro* activation assay was performed using C1R.hMR1 cells as APCs [4] and reporter Jurkat.MAIT cells [73], following published methods as described in Chapter 2 [4]. Culture supernatant samples were prepared as described in Chapter 2 and were collected at the four representative time points for each bacterium, which have been defined in the previous section of this chapter (Chapter 3). After stimulated with bacterial supernatant, MAIT cell activation was measured by the upregulation of CD69 expression in the Jurkat.MAIT cells.

The result is shown in Figure 5. For the positive control 5-OP-RU was used, which is a MAIT activating ligand. While 6-FP is a non-stimulatory ligand of MAIT cells and was used as the negative control. The results of control groups were consistent with previous studies [4, 5] suggesting that the experiment was a valid one.

With the experiments controlled, it was then demonstrated that all three bacteria are capable of producing MAIT-activating ligands, as their supernatants were able to activate Jurkat.MAIT cells, though to different magnitudes. Secondly the individual bacteria appeared to accumulate most activating ligand at various time points: *E. coli* and *K. pneumoniae* at 4 hours, and *S. aureus* at 12 hours post re-inoculation, which is surprising. It was expected that the late log-phase supernatant should elicit the most MAIT cell activation, as a speculated combined result from relatively large number of bacteria and active metabolism. Nevertheless, the best sampling times for each bacteria species have been set for future experiments.

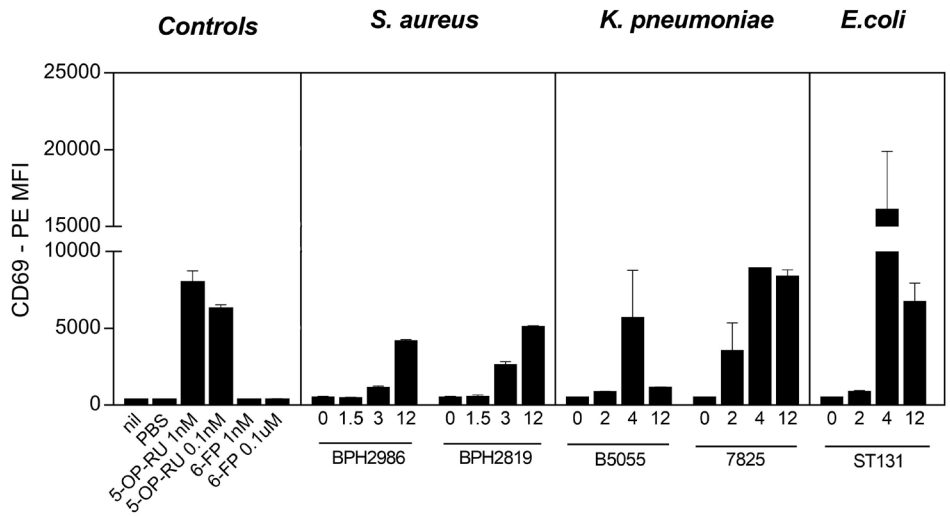


Figure 5 Bacterial supernatant activated Jurkat.MAIT cells *in vitro* as assessed by CD69 expression.

Jurkat.MAIT (10^5) and C1R.MR1 (10^5) cells were co-incubated for 16 hrs at 37°C with 5% CO_2, with supernatant (SN) (20 µl) from bacterial culture prepared at indicated time points (0, 1.5/2, 3 /4 or 12 hrs post re-inoculation), or 5-OP-RU, 6-FP or PBS for controls, in a total volume of 200 µl RPMI/well supplemented with 10% FCS and appropriate antibiotics. Jurkat.MAIT cell activation was measured by up-regulation of CD69. Labelling under the lines indicate specific strains assayed. Data show mean CD69 expression of MAIT cells using fluorescence intensity (MFI), Mean ± range of duplicate wells. Experiment performed in duplicate wells on two separate occasions with similar results.

During bacterial *in vitro* growth, it was unknown whether the bacteria-derived antigens were released passively, actively or upon bacterial cell death. To detect antigens in another way, the bacteria were lysed by sonication as described in Chapter 2, and utilizing the same cellular activation system, the resultant lysates were tested for MAIT cell antigens within bacterial cells. The three bacteria were lysed at their stationary phase (12 hrs after inoculation), by which time the bacteria number should be higher and less fluctuant than the log phase. As can be seen from Figure 6, lysates of *K. pneumoniae* and *E. coli* both activated Jurkat.MAIT cells *in vitro*, whereas CD69 upregulation in Jurkat-MAIT cells was not detected after incubation with the lysate from either strain of *S. aureus* tested.

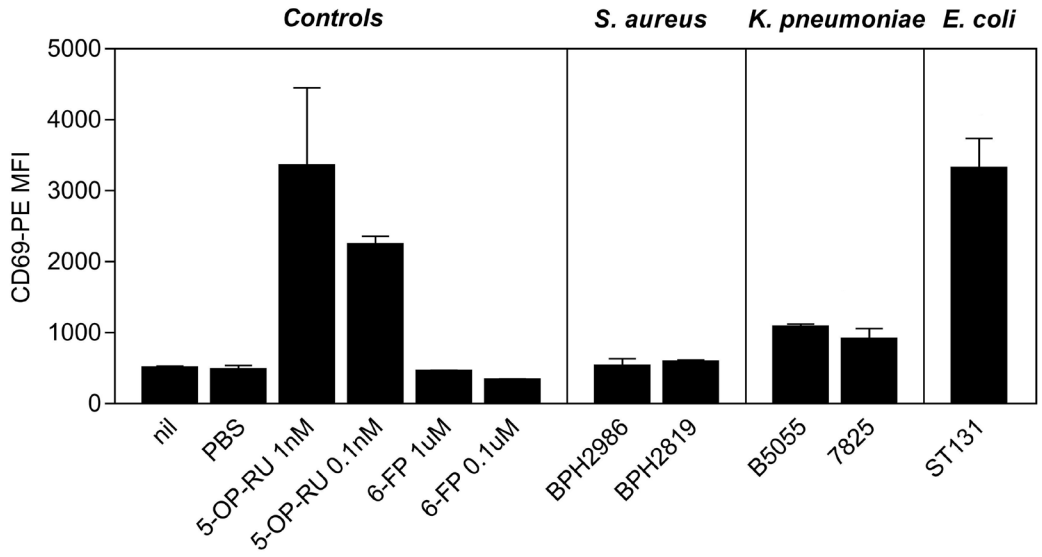


Figure 6 Bacterial lysate activated Jurkat.MAIT cells *in vitro* as assessed by CD69 expression.

Jurkat.MAIT (10^5) and C1R.MR1 (10^5) cells were co-incubated for 16 hrs at 37°C with 5% CO_2, with bacterial lysate (20 µl) prepared at 3 hrs post re-inoculation, or 5-OP-RU, 6-FP or PBS for controls, in a total volume of 200 µl RPMI/well supplemented with 10% FCS and appropriate antibiotics. Jurkat.MAIT cell activation was measured by up-regulation of CD69. Labelling indicate specific strains assayed. Data show CD69 expression of MAIT cells using MFI, Mean ± range of duplicate wells. Experiment performed in duplicate wells on two separate occasions with similar results.

Overall, these results show that *S. aureus*, *K. pneumoniae* and *E. coli* were all capable of stimulating MAIT cells *in vitro*. This suggests that the three clinically important bacteria may activate MAIT cells and become potential targets of MAIT cells when they infect humans and mice. The next section, therefore, seeks to optimize *in vivo* mouse infection models and discuss the MAIT cell response upon infection with the three bacteria.

3.2.3 K. pneumoniae fails to induce a productive but non-lethal intraperitoneal infection in C57BL/6 mice.

Clinically, *Klebsiella pneumoniae* is a common causative organism of intraperitoneal infection, accounting for ~24% of the peritonitis cases [145, 146]. This study aimed to establish an *in vivo K. pneumoniae* peritonitis model and investigate the protective role of MAIT cells against this particular bacterial peritonitis.

To induce a potentially robust MAIT cell response, the bacterial infection should be productive and severe without killing the mice. Therefore, the dose of infection needs to be optimized in the first place. For this purpose, C57BL/6 mice were infected with two strains of *K. pneumoniae* of different virulence: B5055 and 7825 (Figure 7 and personal communication, R. Strugnell), via the intraperitoneal (i.p.) route, and 10 fold different infection doses were tested, which are 10, 100 and 1000 colony forming units (CFU) for strain B5055, or 100 and 1000 CFU for strain 7825. This was to establish the "optimal" infection dose, which was defined as giving least adverse health impact on the mice but inducing a robust, reproducible MAIT cell response. The results are shown in Figure 7.

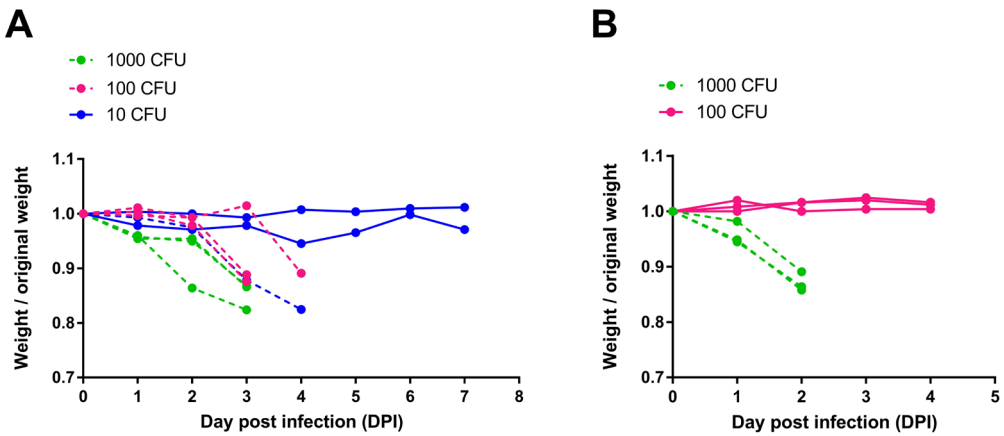


Figure 7 Weight change of mice following intraperitoneal infection with *Klebsiella pneumoniae.*

C57BL/6 mice were intraperitoneally infected with one of two strains of *K. pneumoniae*, A) B5055 and B) 7825 at 1000 CFU (green), 100 CFU (pink) and 10 CFU (blue), and the body weight of mice was monitored after infection. The Y-axis shows the body weight of mice after infection as a proportion of the original weight. The X-axis shows days post-infection. Each line refers to an individual mouse, and dotted lines indicates the mouse reached a humane end point and was killed. The experiment was repeated twice with 3 mice per dose group per strain and had similar results.

Firstly, it appeared rather difficult to establish an optimal dose for *K. pneumoniae.* In this study, weight loss in mice was assumed and adopted as a primary indication of productive infections to avoid extensive CFU analyses of organs. While with the doses tested, *K. pneumoniae* did not manifest an infection at a low dose, or the bacteria killed the mice when using a higher dose. For both stains of *K. pneumoniae*, no mouse was found to recover from a productive i.p. infection (as indicated by weight loss) of the bacteria. When C57BL/6 mice were infected with 100 CFU or more bacteria of both strains, the death rate was 100% at 4 days post-infection. *K. pneumoniae* B5055 showed a relatively higher virulence. Specifically, as low as 10 bacteria introduced to the peritoneal cavity led to either death or non-productive infection. For the mice that survived, their body weight kept steady until day 7 when reaching the experiment endpoint, suggesting the infection was not productive in these mice.

Secondly, no increase of MAIT cell absolute number or percentage was found after *K. pneumoniae* infection. MAIT cells were examined in mice showing some ill symptoms (which suggest the mice were infected) on day 3 and in surviving mice on day 7. MAIT number increase was not found in abdominal cavity, liver or spleen. These results suggest that it is difficult to establish an "optimal dose" with this bacterial infection. Due to the low survival rate of mice after infection, this experiment was incomplete, and the findings should be interpreted with caution. Nevertheless, it was decided not to continue working with the current strains of *K. pneumoniae* further for this project."

In summary, the above experiments showed after inoculated with *K. pneumoniae* intraperitoneally, mice ended up with an unpredicted binary fate: they either succumbed to a productive infection or showed no infection. These results suggested that it was difficult to establish an infection model with *K. pneumoniae* for assaying MAIT cell immunity. Therefore, the following sections moved on to trial the other two clinically important bacteria, namely *S. aureus* and *E. coli*.

3.2.4 S. aureus intraperitoneal infection causes local MAIT cell accumulation

S. aureus are opportunistic, easy transmitted bacteria that clinically accounts for skin abscess formations, pulmonary infections and intraperitoneal infections. Next, this study then set out to establish an *S. aureus* peritonitis models for MAIT cell studies., *S. aureus* BPH2819, the *S. aureus* strain that showed the best activation of

Jurkat.MAIT cells according to the results of *in vitro* experiments (Figure 5), was chosen for the following experiments. Intraperitoneal infection was achieved by injecting a sub-lethal dose of 10^7 CFU bacteria to C57BL/6 mice, and this model was assessed thereafter.

It was first found that *S. aureus* caused a mild symptomatic (judged by appearance and responsiveness), but persistent infection in the peritoneal cavity of mice (Figure 8). The bacterial burden during the infection course was studied by CFU analysis. Mice were euthanatized on 1, 2, 3, 5 or 7 days post infection (DPI), and the bacterial loads within the peritoneal cavity (lavage fluid), liver and spleen were determined at each time point. As can be seen from Figure 8, the number of bacteria in the peritoneal cavity and the liver slowly declined after infection, and by 7 days post infection (DPI) with 10^7 CFU, 10^3~10^4 CFU of bacteria were still detectable. The bacterial burden in the spleen was relatively more fluctuant between 2 and 5 DPI, but generally resembled the infection dynamics seen in the liver and the peritoneal cavity. These data suggested that *S. aureus* could not be efficiently cleared by the host within a 7 day time course and elicited a non-lethal infection in the peritoneal cavity and abdominal organs.

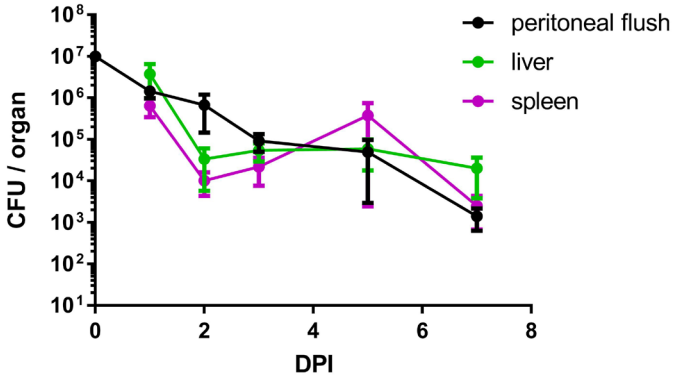


Figure 8 Bacterial load kinetics of C57BL/6 mice during *S. aureus* BPH2819 intraperitoneal infection.

C57BL/6 mice were infected with a sub-lethal dose (10^7 CFU) of *S. aureus* BPH2819 intraperitoneally (i.p.), and the bacterial load from abdominal cavity (black), liver (green) and spleen (purple) were measured by plating out the 200 µl of peritoneal flush or homogenized organ fluid. The Y-axis shows the bacterial load (CFU

(Mean±SEM, n=4). The X-axis shows days post-infection (DPI). The experiment was performed twice with similar results.

The next question addressed was whether MAIT cells participated in the immune response upon *S. aureus* infection. Absolute number and percentage of MAIT cells were examined by flow cytometry analysis (Figure 9A) with MAIT cells gated according to the strategy in Chapter 2 (Figure 3). It was found that the peritoneal cavity MAIT cells significantly increased in number and as a percentage of total T cell at 7 DPI, as shown in Figure 9. On average, approximately 100~200 MAIT cells could be recovered from the peritoneal cavity in a naive C57BL/6 mouse. This number increased to 10^3~10^4 post *S. aureus* i.p. infection. Interestingly, although the bacterial load of liver and spleen were as high as the peritoneal cavity by day 7 (Figure 8), no significant MAIT cell accumulation in these two organs was observed.

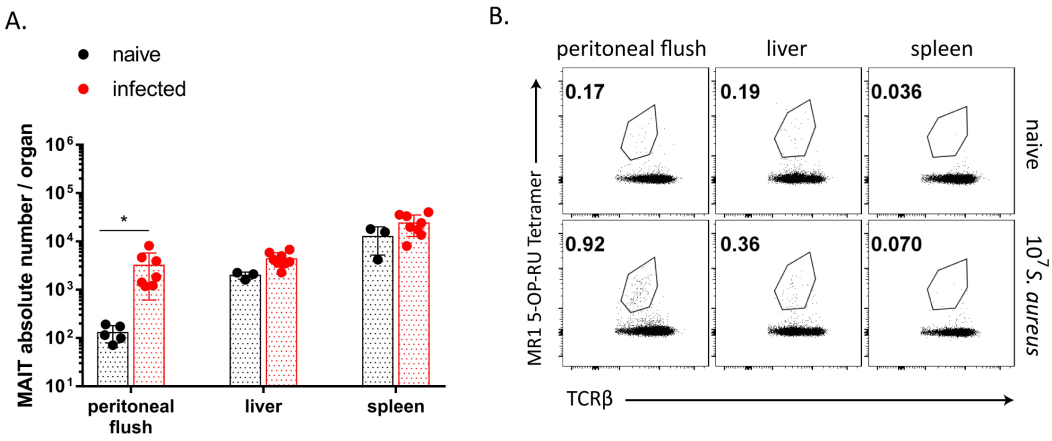


Figure 9 Infection with a clinical strain of *S. aureus* led to MAIT cell expansion in the abdominal cavity, but not in liver or spleen.

C57BL/6 mice were infected with10^7 CFU *S. aureus* BPH2819 intraperitoneally. 7 days post-infection, A) absolute number of MAIT cells (Mean ± SD) in peritoneal cavity, liver and spleen were compared between naïve (black dots) and infected (red dots) mice. MAIT cell numbers significantly increased in the abdominal cavity after infection (*=P<0.05, Student's t-test). B) Representative FACs plots showing percentage of MAIT cells in total αβ T cells. The experiment was performed twice (3~7 mice per group each time) with similar results.

In summary, this set of experiments showed that i.p. infection of mice with *S. aureus* BPH2819 resulted in mild accumulation of MAIT cells in the peritoneal cavity. MAIT cells which are locally enriched in the abdominal cavity could be further analyzed for their potential anti-microbial functions. However, the relatively low absolute numbers (~2000) of MAIT cells even after proper infection would render difficulties when examining functions of MAIT cells in more details (Figure 9).

3.2.5 E.coli ST131 infection induces a significant accumulation of MAIT cells locally and in peripheral organs.

Escherichia coli is a commensal organism of human gut microbiota, but it induces peritonitis when translocated or disseminated to peritoneal cavity, which is commonly seen in liver cirrhosis patients [161, 162]. As a surrogate of the study of human infection, an intraperitoneal infection model of *E. coli* clinical isolate ST131 was optimized in C57BL/6 mice. The infection dose was first optimized. As shown in Figure 10A, mice survived an i.p. infection with 10^6 CFU of *E. coli* ST131 but succumbed to infection with a dose of 10^7 CFU.

To determine the *in vivo* clearance kinetics of *E. coli*, bacterial loads recoverable from multiple organs at various time points were analyzed over 7 days post infection (Figure 10B). The results suggest that *E. coli* replicated *in vivo* in the early phase of infection, specifically before 1 DPI in the peritoneal cavity and up to 3 DPI in the liver.

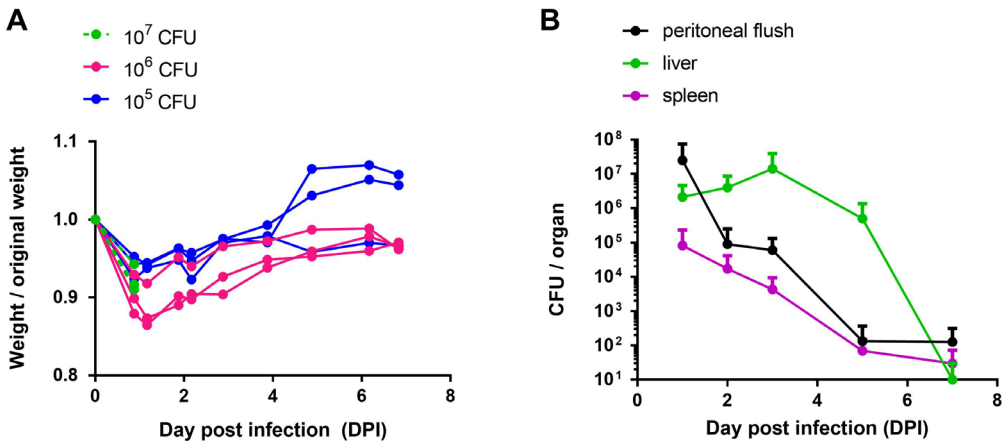


Figure 10 Body weight and bacterial load kinetics of C57BL/6 mice during *E. coli* infection.

C57BL/6 mice were infected i.p. with 10^7 (green), 10^6 (pink) and 10^5 (blue) CFU of *E. coli* ST131 and their body weight (A) was monitored after infection. Each line represents one experimental mouse. Dotted line indicates the mouse reached a humane end point after infection and was killed. (B) 10^6 CFU of *E. coli* ST131 was introduced to the C57BL/6 mice peritoneal cavity by i.p. injection. Infection kinetics shows the bacterial load (Mean + SEM, n=4) of the abdominal cavity (black), liver (green) and spleen (purple). The experiment was repeated three times with similar results.

The accumulation of MAIT cells induced by *E. coli* peritonitis was analysed similarly as described in chapter 2. Representative FACS plots are depicted in Figure 11, and statistical analysis is shown in Figure 12. This data set indicates that *E. coli* infection elicited significant MAIT cell accumulation in all of the organs assessed, including the peritoneal cavity, liver and spleen. Moreover, MAIT cells amassed in a dose dependent manner, as their percentage was higher in mice challenged with the sub-lethal dose in this model (2x10^6 CFU *E. coli*) (Figure 11).

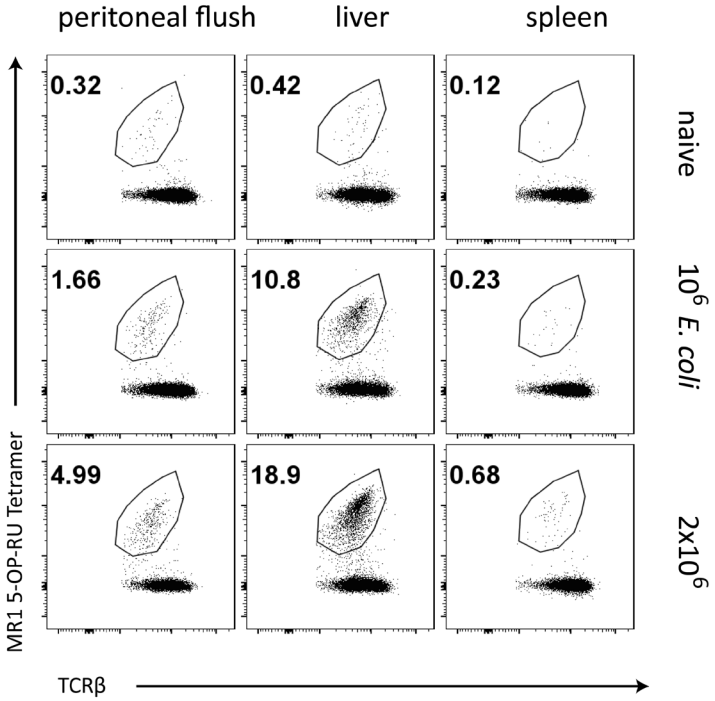


Figure 11 Representative FACS plots demonstrate *E. coli* infection boosted MAIT cells in affected organs.

C57BL/6 mice were infected with 10^6 or 2x10^6 CFU *E. coli* ST131 intraperitoneally. At 7 days post infection, mice were killed, and cells prepared from indicated organs were analysed for MAIT cell accumulation. The numbers in the FACS plots represent the percentage of MAIT cells against total αβ T cells. The experiment was repeated twice with similar results.

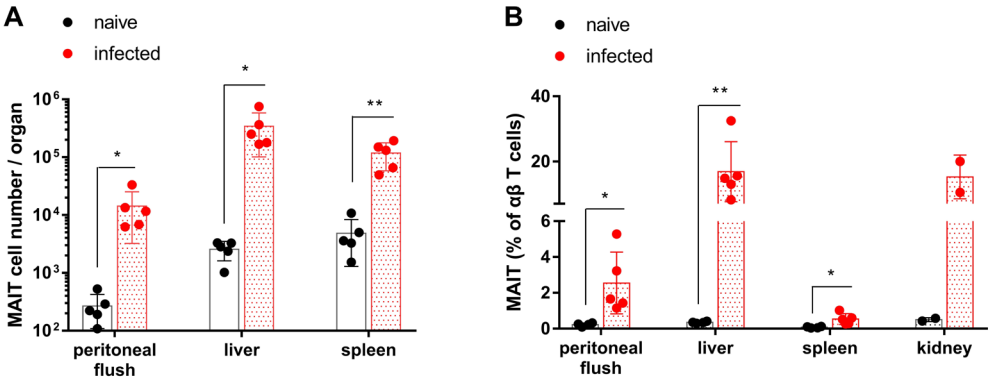


Figure 12 *E. coli* ST131 intraperitoneal infection caused significant accumulation of MAIT cells in the peritoneal cavity, liver, spleen and kidney.

C57BL/6 mice were infected with 10^6 CFU *E. coli* ST131 intraperitoneally. 7 days post-infection, mice were killed and MAIT cell accumulation from indicated organs was analysed. A) Absolute number and B) percentage of MAIT cells in peritoneal cavity, liver, spleen and kidney were compared between naïve (black dots) and infected (red dots) mice. Mean ± SEM for peritoneal flush, liver and spleen, Mean ± range for kidney. The experiment was repeated three times with similar results. (*=P<0.05, **=P<0.01, Student's t-test)

3.2.6 MAIT cell deficient Mr1$^{-/-}$ mice are more susceptible to E. coli peritonitis.

Next this study moved on to elucidate the role of MAIT cells during *E. coli* infection. To test if MAIT cells take an active part in clearing *E. coli* in the process of acute peritonitis, mouse survival and bacterial loads were compared between wild type C57BL/6 mice (MAIT proficient) and *Mr1*$^{-/-}$ mice (MAIT cell deficient). Age- and gender- matched *Mr1*$^{-/-}$ mice and C57BL/6 mice were infected with 10^6 CFU of bacteria via i.p injection. The bacterial loads of within the peritoneal cavity and other organs were determined on day 1, 3 and 5 post infection, and were compared between the two strains of mice.

The most dramatic observation was from the survival rates. 5 days after infection, MAIT-containing C57BL/6 mice survived significantly better (survival rate: 100%) than the MAIT deficient *Mr1*$^{-/-}$ mice (80%) (Figure 13B). Furthermore, the surviving *Mr1*$^{-/-}$mice had a higher bacterial burden in the peritoneal cavity on day 3 post-infection compared to wild type C57BL/6 mice (Figure 13C). Surprisingly, it was found that *Mr1*$^{-/-}$ mice appeared to regain weight more rapidly from the second day onward after infection (Figure 13A), though the difference was not statistically significant. Taken together, these results indicate that in the early stage of infection, MAIT cells in the peritoneal cavity were important in controlling *E. coli* infection, while they were also possibly contributing to a severer immune response that leads to more sustained weight loss in infected mice.

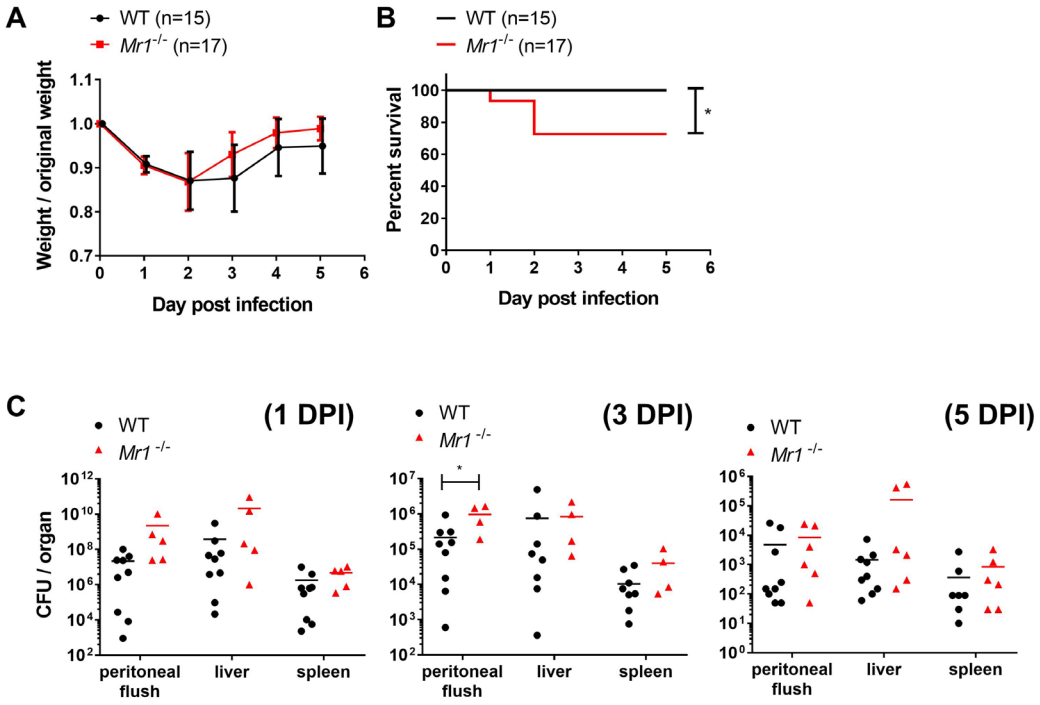


Figure 13 *E. coli* peritonitis was less well controlled in MAIT cell deficient *Mr1*$^{-/-}$ mice.

Age- and gender- matched C57BL/6 (wildtype, WT) and *Mr1*$^{-/-}$ mice were infected with 10^6 CFU *E. coli* ST131 intraperitoneally. A) Weight changes and B) survival rates were compared between two strains of mice. Statistic: Log-rank (Mantel-Cox) test, *P<0.05. C) Bacterial load was detected in peritoneal cavity, liver and spleen for both strains of mice, and was determined on 1, 3 and 5 days after infection. Horizontal lines indicate the median. *=*P*<0.05, unpaired t-test.

3.2.7 MAIT cells are skewed to a MAIT-1 (Th1-like) phenotype during E. coli infection

Literature suggest that MAIT cells bear functional plasticity, and in response to different pathogens, can be polarized to a more beneficial phenotype and produce distinct cytokines [163]. Specifically, previous research suggested that murine MAIT cells can be divided into two distinct subsets, either MAIT-1 or MAIT-17 [93, 101, 164], defined by the expression of transcription factors RORγt and T-bet. Similar to Th1 and Th17 subsets of T-helper cells, the MAIT-1 subset is characterized by T-bet$^+$ phenotype and interferon γ (IFN-γ) production, while the MAIT-17 subset is a RORγt$^+$, interleukin-17 (IL-17) producing MAIT cell population [164]. To ascertain MAIT cells' potential protection mechanisms during acute peritonitis by *E. coli*, this study explored the profiles of transcription factors and cytokines of MAIT cells activated during *E. coli* ST131 peritonitis.

The profile of transcription factors T-bet and RORγt was compared between naïve and stimulated MAIT cells (Figure 14 A). In naïve mice, it was first found that MAIT cells isolated from the peritoneal cavity, liver, spleen and kidney, possessed different transcription factor profiles. Similar to lung, thymus and lymph node MAIT cells [86, 165], peritoneal cavity MAIT cells in naïve mice were mostly (> 89%) RORγt positive, indicative of a MAIT-17 profile (Figure 14A). This result contrasts to the transcription factor profile of MAIT cells from other abdominal organs (Figure 14A). T-bet single positive (MAIT-1 profile) and RORγt single positive MAIT-17 subsets were observed at approximately 1:1 ratio in the kidney and spleen, while MAIT-1 was the dominant phenotype of MAIT cells in the liver and kidney (Figure 14A). These data suggest that MAIT cells from different organs have different transcription factor profiles.

Interestingly, *E. coli* stimulated MAIT cells showed different expression pattern of transcription factors compared to naïve MAIT cells. Despite the organ specific transcription factor profiles of MAIT cells in naïve mice, 7 days after *E. coli* peritonitis, T-bet$^+$ MAIT-1 cells became dominant in the all the organs tested, including peritoneal cavity, kidney, liver and spleen (Figure 14A and B). In particular, this phenotype polarization was most significant in peritoneal cavity MAIT cells, in which T-bet$^+$ MAIT percentage increased from 5% to 80% (Figure 14B). A population of T-bet$^+$RORγt$^+$ (MAIT-1/MAIT-17 profile) MAIT cells was also observed and showed increased percentage (from 6% to 16%) after *E. coli* infection (Figure 14A).

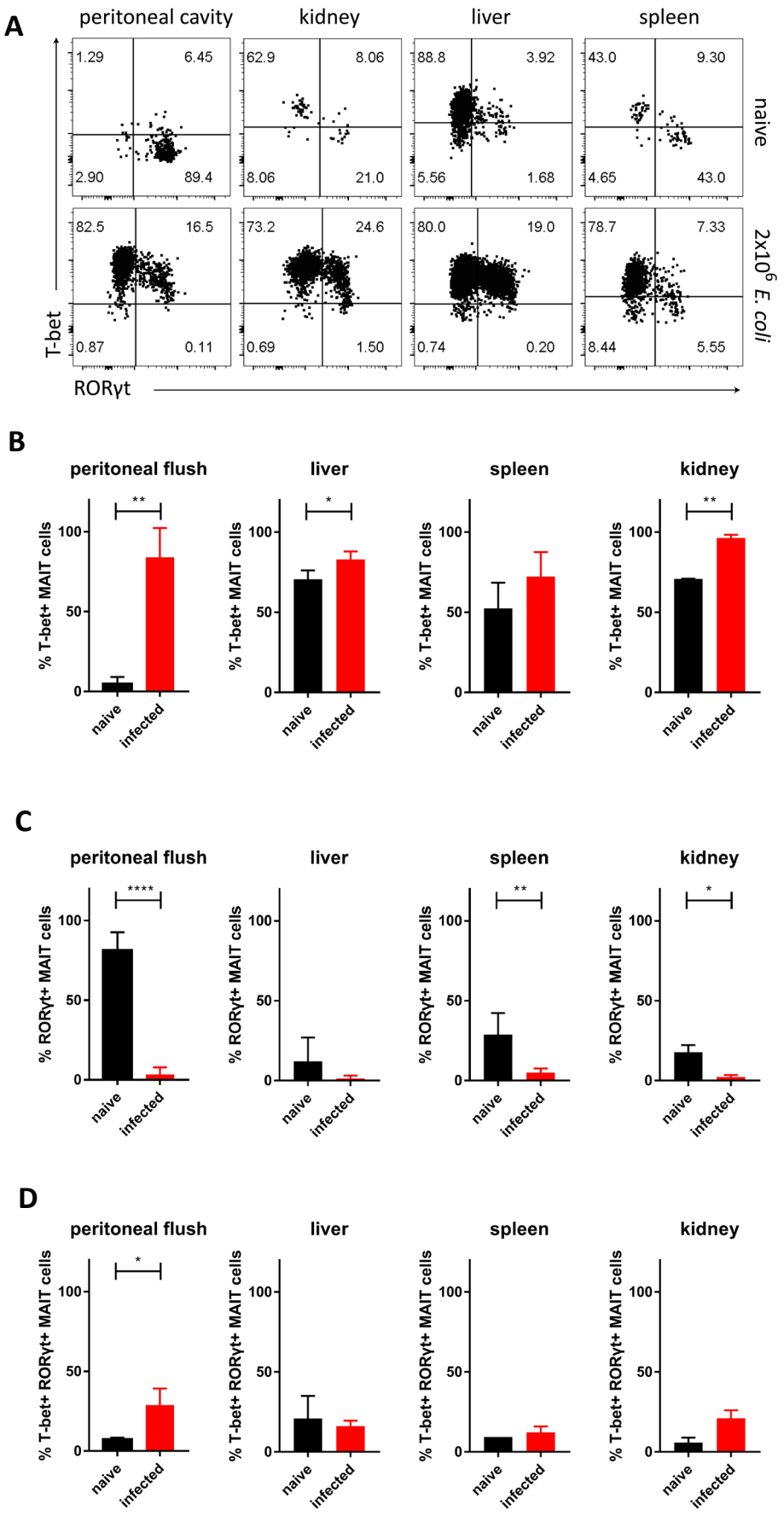

A
peritoneal cavity
kidney
liver
spleen
naive
2x10^6 E. coli
T-bet
RORγt
1.29
6.45
2.90
89.4
62.9
8.06
8.06
21.0
88.8
3.92
5.56
1.68
43.0
9.30
4.65
43.0
82.5
16.5
0.87
0.11
73.2
24.6
0.69
1.50
80.0
19.0
0.74
0.20
78.7
7.33
8.44
5.55
B
peritoneal flush
liver
spleen
kidney
% T-bet+ MAIT cells
naive
infected
C
% RORγt+ MAIT cells
D
% T-bet+ RORγt+ MAIT cells

Figure 14 Transcription factor profiling of MAIT cells during acute *E. coli* ST131 peritonitis.

A) Representative FACS dot plots showing the T-bet and RORγt expression profiles of gated MAIT cells from naive C57BL/6 mice (top panels) and mice infected with $2x10^6$ CFU *E. coli* ST131 (bottom panels). Figure shows MAIT cells only ($CD45.2^+$, $7AAD^+$, $CD19^-$, $TCR\beta^+$, MR1-5-OP-RU $tetramer^+$ population).

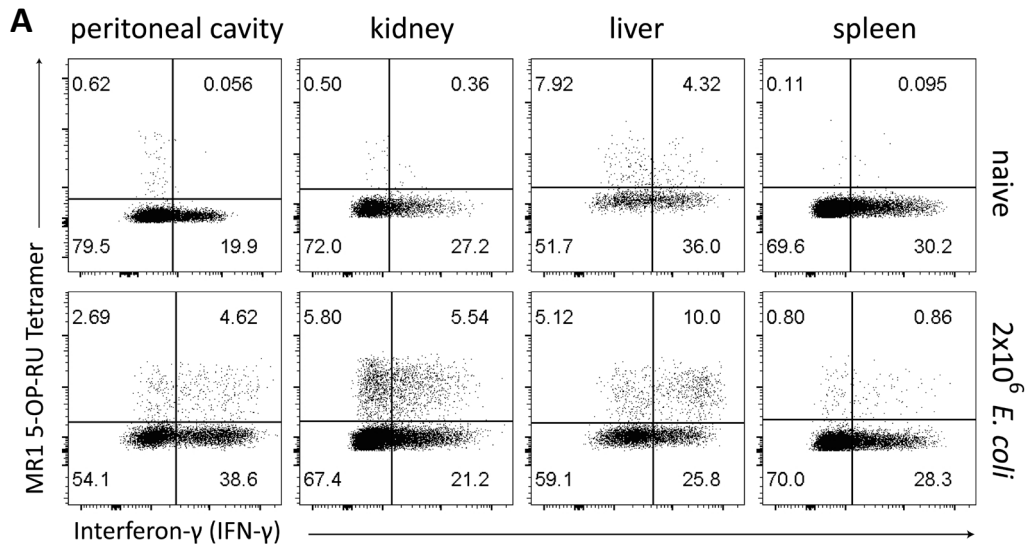
B) Percentage of T-bet^+ MAIT cells in naive (n=3) and infected mice (n=5) (Mean + SD). Statistic: unpaired t-test, *=$P<0.05$, **=$P<0.01$. The experiment was repeated twice with similar results.

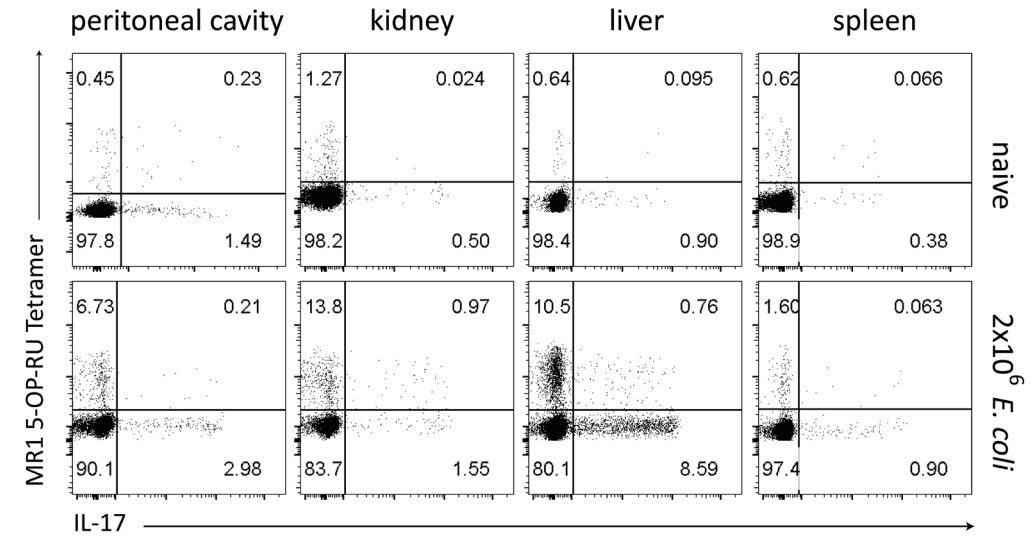
C) Percentage of RORγt^+ MAIT cells in naive (n=3) and infected mice (n=5) (Mean + SD). Statistic: unpaired t-test, *=$P<0.05$, **=$P<0.01$. The experiment was repeated twice with similar results.

D) Percentage of T-bet^+ RORγt^+ MAIT cells in naive (n=3) and infected mice (n=5) (Mean + SD). Statistic: unpaired t-test, *=$P<0.05$, **=$P<0.01$. The experiment was repeated twice with similar results.

To further investigate the roles played by the phenotypic polarized MAIT cells, the production of two cytokines, IFN-γ and IL-17, which represent the key cytokines for MAIT-1 and MAIT-17 populations respectively [164], were analysed by intracellular staining and flow cytometry. In accordance with their predominantly MAIT-1 phenotype, MAIT cells from peritoneal cavity produced a large amount of IFN-γ but little IL-17 on day 7 after *E. coli* i.p. infection, as shown in Figure 15. It was observed that IFN-γ positive MAIT cells significantly increased in proportion (from 5% to 60%), when compared to that of naive mice. In contrast, no difference was found when comparing IL-17 production by MAIT cells from naïve and infected mice, with MAIT cells from both groups of mice produced low levels of IL-17.

In summary, from the transcription factor and cytokine analysis, these data suggest that MAIT cells were driven to a MAIT-1 phenotype during acute peritoneal infection by *E. coli* ST131. Moreover, this MAIT-1 phenotype was characterized by expression of transcription factor T-bet and production of IFN-γ.


A
peritoneal cavity
kidney
liver
spleen
0.62
0.056
79.5
19.9
0.50
0.36
72.0
27.2
7.92
4.32
51.7
36.0
0.11
0.095
69.6
30.2
naive
2.69
4.62
54.1
38.6
5.80
5.54
67.4
21.2
5.12
10.0
59.1
25.8
0.80
0.86
70.0
28.3
2x10^6 E. coli
MR1 5-OP-RU Tetramer
Interferon-γ (IFN-γ)


B
peritoneal cavity
kidney
liver
spleen
0.45
0.23
97.8
1.49
1.27
0.024
98.2
0.50
0.64
0.095
98.4
0.90
0.62
0.066
98.9
0.38
naive
6.73
0.21
90.1
2.98
13.8
0.97
83.7
1.55
10.5
0.76
80.1
8.59
1.60
0.063
97.4
0.90
2x10^6 E. coli
MR1 5-OP-RU Tetramer
IL-17

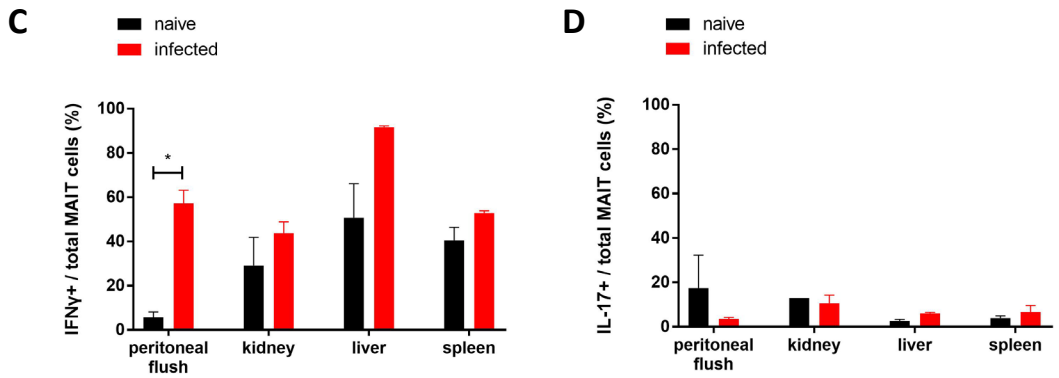

C
naive
infected
IFNγ+ / total MAIT cells (%)
100
80
60
40
20
0
*
peritoneal flush
kidney
liver
spleen
D
naive
infected
IL-17+ / total MAIT cells (%)
100
80
60
40
20
0
peritoneal flush
kidney
liver
spleen

Figure 15 Cytokine profile of MAIT cells during acute peritonitis by *E. coli* ST131.

A) and B). αβ T cells were analyzed from naïve and infected C57BL/6 mice at 7 days post intraperitoneal $2x10^6$ CFU *E. coli* ST131 infection. Representative flow cytometry plots showing the intracellular cytokine staining for A) IFN-γ and B) IL-17 of αβ T cells from the peritoneal cavity, kidney, liver and spleen. MAIT cells are shown in the top quadrants of each plot as the MR1 5-OP-RU^+ population. The number represents the percentage of total αβ T cells.

C) and D). MAIT cells from naive mice (n=2) and infected mice (n=5) were gated and analyzed for IFN-γ and IL-17 expression respectively. Percentage of IFN-γ (C) or IL-17 (D) expressing MAIT cells are depicted before and after *E. coli* infection. (Mean ± SD). *=$P<0.05$, unpaired t-test.

The experiment was repeated twice with similar results.

3.3 Discussion

In studies of this chapter, bacterial peritonitis models were trialed in laboratory strains of mice with three clinically important bacteria: *K. pneumoniae*, *S. aureus* and *E. coli* [166]. The three bacteria showed differences in virulence (*in vivo*) and MAIT cell activation (*in vitro* and *in vivo*) and, and consequently, not all of them are suitable for inducing peritonitis and studying MAIT cells under laboratory conditions.

Utilizing a pre-established *in vitro* assay, this study first found that the SN of all the three bacteria could stimulate MAIT cell activation *in vitro* (Figure 5). As suggested by previous studies, the stimulatory ligands (5-OP-RU) of MAIT cells are directly derived from a riboflavin biosynthesis intermediate, 5-A-RU [5]. *K. pneumoniae*, *S. aureus* and *E. coli* all bear a riboflavin biosynthesis pathway, and in conjunction with the present result, the bacterial capacity in sitmulating MAIT cell responses was suggessted. Due to the lack of MR1 control groups using either anti-MR1 monoclonal antiobdies or *Mr1*$^{-/-}$ cell lines, this study has not elucidated if the MAIT cell activation was induced in a TCR dependent manner. Nevertheless, it seemed that log phase SN was not as efficient in stimulating MAIT cells compared to that of stationary phase, suggesting that the bacterial growth phase influenced the amount of 5-A-RU derived ligands in the SN. This has been observed with all the three bacteria, and was especially obvious with *S. aureus.* Although the production of 5-A-RU has been less studied, the biosynthesis of end-product (riboflavin) has been well characterized. A promotion of riboflavin biosynthesis has been linked to a decreased growth rate (i.e. the transition from log to exponential phases) [159, 160], which support the present result. Also, our finding is consistent with that of Schmaler *et al.* (2018) who reported that MAIT cells secreted more IFN-γ when stimulated with a lysate of *E. coli* at stationary phase than at log phase [167]. Therefore, this study is consistent with previous observations and suggest that the bacteria at stationary phase are potentially more efficient in stimulating MAIT cells.

Interestingly, this study also found that the SN and lysate of three bacteria showed difference in ability of stimulating MAIT cells. While *E. coli* lysate at stationary phase still harboured a significant amount of MAIT-activating ligand, *K. pneumoniae* and *S. aureus* appeared to shed ligands extracellularly (into the supernatant) and their lysate did not induce activation of MAIT reporter cells. However, the MAIT cell activation stimulated by bacterial SN and lysate from the same sample should be compared in one experiment to solidify the current conclusion. Given that the stimulatory ligand

5-OP-RU is chemically unstable and was expected to accumulate as well as degrade over the time course of *in vitro* bacterial growth [5], the results should be interpreted with caution and await explanations from further investigations.

While *S. aureus* and *E. coli* successfully induced bacterial peritonitis in mice, *K. pneumoniae* did not, with the same experimental procedures. Specifically, this study found that intrperitoneal inoculation of 1000 CFU *K. pneumoniae* would lead to 100% mortality in C57BL/6 mice. This outcome is contray to that of Georgel *et al*. (2011), who set up an intraperitoneal infection model with $2x10^8$ CFU of *K. pneumoniae* with <20% mortality in C57BL/6 mice [143]. A possible explanation for this discrepancy is that the two studies used different strains of *K. pneumoniae* which differ in virulence in mice. The two strains of *K. pneumoniae* used in this study are clinical strains but mouse adpated, wheras Georgel *et al*. (2011) used a gene-modified luminescent strain, which could be less pathogenic to mice. Another interesting finding of this study is that some mice showed no weight loss after 10 or 100 CFU *K. pneumoniae* infection. One possible explanation is that *K. pneumoniae* secretes polysaccharide when cultured *in vitro*, which is sticky and made inoculum preparation inaccurate [168]. Given the infection dose was as low as 10 CFU in 200 µl, it was probable that bacteria were not injected into the peritoneal cavity of some mice, resulting in non-productive infections in some mice. Nevertheless, CFU analyses of organs from infected mice will be needed for determining whether individual mouse was infected or not, and further technical optimization will be needed for improving accuracy of inoculum preparation with *K. pneumoniae*.

This study successfully established two peritonitis models with *S. aureus* and *E. coli*. The infection doses were optimised to be 10^8 CFU *S. aureus* BPH2815 and 10^6 CFU *E. coli* respectively, which induced serious infections without killing the mice. The two doses of infection accord with published paper despite differences in the actual strains of *S. aureus* and *E. coli* used [169, 170]. Although *S. aureus* infection persisted for over 7 days in the peritoneal cavity (Figure 8), it did not to induce a significant response of MAIT cells *in vivo* (Figure 9). By contrast, *E. coli* infection was less persistent in the peritoneal cavity (Figure 10B), but caused a robust expansion of the MAIT cell population in multiple organs (Figure 11, 12). This result is consistent with the *in vitro* assay results, that MAIT cells became highly activated with *E. coli* SN/lysate but only mild activation was manifested in the context of *S. aureus* SN/lysate (Figure 5, 6). The *in vitro* assay results suggested there are differences between the two bacteria in MAIT cell ligand production, which might be reflected by the MAIT cell responses *in vivo*.

For the first time, this study shows that MAIT cells robustly amassed in the peritoneal cavity and other organs in response to *E. coli* peritonitis (Figure 12), suggesting their participation in the anti-bacterial immunity. A lower mortality and lower bacterial burden in surviving mice 3 days after infection were observed in C57BL/6 mice in comparison with $Mr1^{-/-}$ mice (Figure 13). Similarly, Le Bourhis *et al.* (2010) showed that, compared with the $Mr1^{+/+}$ equivalent, $Mr1^{-/-}$ Vα19*i*Tg mice had higher splenic bacterial burdens after *E. coli* peritonitis [75]. These results suggested that MAIT cells in the peritoneal cavity played a protective bridging role between innate and adaptive immunity and helped control the bacterial replication during the early stage of the acute peritonitis by *E. coli*. For any clinical application, it would be interesting to know if vaccination (boosting) of MAIT cells prior to infection will further amplify this MAIT cell-mediated protection [101].

The potential mechanisms of MAIT cell protection was also investigated in this study. During acute peritonitis by *E. coli,* MAIT cells with T-bet$^+$ (MAIT-1) phenotype and IFN-γ production significantly increased in percentage (Figure 14,15), suggesting that MAIT cells were activated and contributed to the cytokine response. This result corroborates several *in vitro* infection cellular studies, in which MAIT cell production of IFN-γ has been reported when stimulated by *E. coli* [8, 131]. In addition, IFN-γ is well-known for its importance in immnue responses when defending against bacterial infection [171, 172], and MAIT cell IFN-γ production was shown to be important in their contribution to clearance of *Legionella longbeachae* lung infection [101]. In the context of *E. coli*, the importance of IFN-γ has also been suggested. IFN-γ promotes phagocytosis of bacteria and reverses monocyte paralysis [173, 174]. The suppression of IFN-γ production is a critical pathogenesis of Enterohemorrhagic *E. coli* (EHEC) infection [175]. Given that these IFN-γ producing MAIT cells potentially contributed to controlling *E. coli* peritonitis, future studies could further explore the relative role of MAIT source IFN-γ compared to that of other immune cells and the necessity of MAIT cells in clearing bacteria specifically in the peritoneal cavity. Also, while this study has reported different transcription factor profiles between naïve and *E. coli* stimulated MAIT cells, it is unclear whether the MAIT-1 cells after infection were from selective expansion, conversion from local MAIT-17 cells or recruited from the liver, where most MAIT-1 cells observed. Therefore, a future study could assess the detailed reasons that explain this change of MAIT cell phenotype.

Overall, the studies mentioned in this chapter helped fill the gap between clinical bacterial infection and laboratory MAIT cell research. Firstly, this project contributes to establishing clinically relevant bacterial infection models in mice. Secondly, this

research, which addressed the anti-microbial properties of MAIT cells, suggests potential MAIT cell-based treatment to clinical infections in the future.

Chapter 4: Defining antigen presenting cell subsets for MAIT cell activation during *Francisella tularensis* infection

4.1 Introduction

Francisella tularensis, the causative organism of zoonotic tularemia, is a Gram-negative, facultative intracellular bacterium and is highly pathogenic to human and mice [176]. When inhaled to the respiratory tract, *F. tularensis* causes respiratory tularemia in human and potentially progresses to pneumonic tularemia [177, 178]. It has been reported that ten bacteria are sufficient to cause respiratory tularemia in humans [179], and without timely treatment, the infection can become lethal in 30-60% patients [180]. Therefore, it is of vital importance to investigate the immune responses to this bacterium.

MAIT cells have previously been shown to play an important role in controlling pulmonary infection of *F. tularensis* in a mouse pulmonary infection model [140]. Activation of MAIT cells have been detected upon the infection of *F. tularensis* both *in vivo* and *in vitro* [40, 51]. Utilizing an attenuated Live Vaccine Strain (LVS) of *F. tularensis*, it has been demonstrated in a condition of pulmonary infection in mice that the bacteria induce MAIT cell activation *in vivo*, characterized by the production of IFN-γ, TNF and IL-17A [140]. However, this study did not show how MAIT cells were activated upon *F. tularensis* infection. Another *in vitro* study by Jesteadt *et al.* found that MAIT cells secreted IFN-γ when co-cultured with *F. tularensis* LVS-infected macrophages [181], but whether MAIT cells could be activated by other antigen presenting cells was not investigated. Given the complexity of the mammalian immune system, it is still unclear how MAIT cells are recruited and activated upon an *in vivo F. tularensis* infection.

Therefore, this study set out to define the APC subsets capable of activating MAIT cells during *F. tularensis* infection. Based on a pulmonary infection model of *F. tularensis* LVS established by the host lab, the first analysis investigated the bacterial internalization, which was assumed to happen in APCs that present antigens of this intracellular bacterium. The APC subsets were then assessed in an optimized *ex vivo* cellular system, and their ability of inducing MAIT cell activation was explored.

4.2 Results

4.2.1. Bacteria were harboured by APCs after F. tularensis LVS intranasal infection of mice.

F. tularensis is an intracellular bacterium and as shown by previous studies, replicates in a variety of types of cells including macrophages and monocytes [182-184]. Upon invasion of *F. tularensis* during infection, APCs potentially capture the bacteria derived antigens (e.g. 5-OP-RU) and transport the antigens in conjugation with MR1 molecules to the cell surface. MAIT cells thus could recognize the infected cells and become activated. Therefore, to assess the antigen presentation of APCs during *F. tularensis* pulmonary infection, it is desirable to examine if APCs harbour bacteria after inoculation.

Four subsets of APCs, including alveolar macrophages, neutrophils, monocytes and $CD103^+$ dendritic cells (DC), were suggested to be important by previous *in vitro* studies [140, 185, 186], therefore were chosen for this study. $Rag2^{-/-}$ mice lack expression of the RAG recombinases and thus lack both T and B cells. It was previously shown in the host lab that T and B cell presentation on MR1 was not important for MAIT cell activation in this model (unpublished personal communication, Z. Chen). Therefore, $Rag2^{-/-}$ mice were chosen as APC donors to simplify cell sorting process and to avoid potential contamination by T and B cells. Lung APCs were sorted 3 days after intranasal infection of mice with 150 CFU *F. tularensis* LVS (Figure 16). The percentage (Figure 17) of APCs in total lung cells and intracellular bacterial loads (Figure 18) of the four subsets of APC were tested.

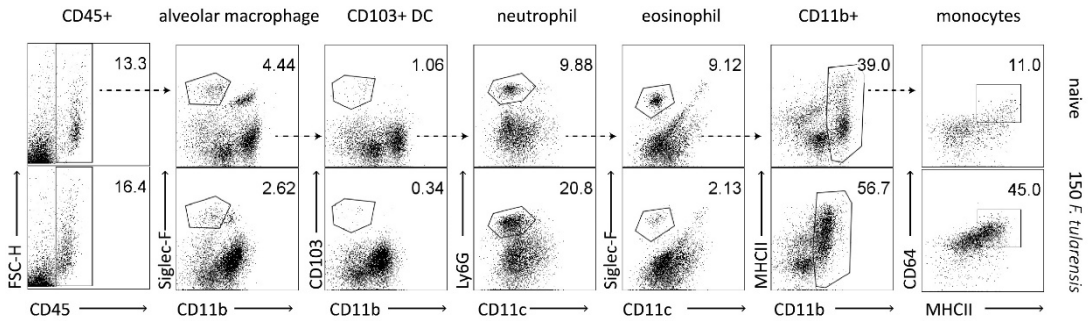


Figure 16 Gating strategy to identify lung APC subsets.

The upper panel shows naïve mice, the lower shows infected mice. Names of gated cells are displayed above each figure. Percentage is shown in the top right corner. Dashed lines indicate the population is further analysed in the next panel. Lung cells were first determined by the forward and side scatter profile (not shown). Singlets were selected and the $CD45^+$ population gated. CD11b and Siglec-F expression were used to identify alveolar macrophage as a $CD11b^{mid}$, Siglec-F^+ population. $CD103^+$ dendritic cells were then further identified and gated by the expression of CD103. The relative expression of CD11c and Ly6G was adopted to identify $Ly6G^+$ neutrophils, CD11c and Siglec-F to identify Siglec-F^+ eosinophils. $MHCII^+$ $CD64^+$ monocytes were then determined on $CD11b^+$ populations.

It was first observed that the pulmonary infection of *F. tularensis* incurred a significant increase of APCs (percentage of $CD45^+$ over total cells isolated) in the lungs (Figure 17A). Neutrophils and monocytes contributed most to the cell influx (Figure 17B) as both subsets increased significantly. By contrast, the percentages of alveolar macrophage and dendritic cell remained unchanged, which is consistent with a published report [183].

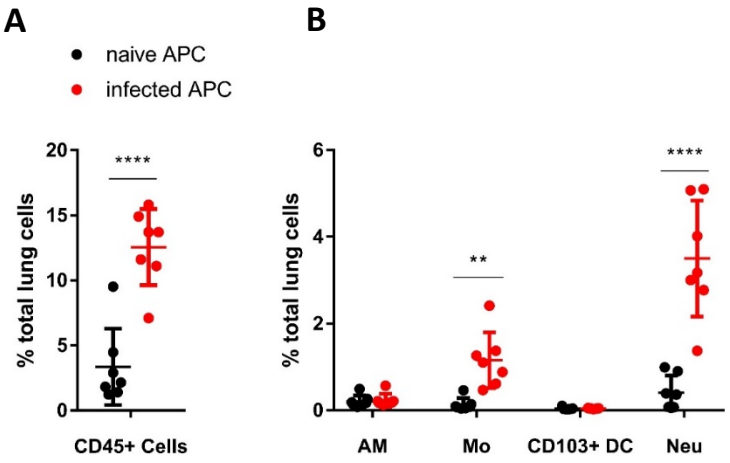


Figure 17 The percentage of APCs increased after *F. tularensis* LVS intranasal infection.

Rag2$^{-/-}$ mice were infected with 150 CFU *F. tularensis* LVS intranasally. Percentage of lung APC subsets over total lung cells were analysed 3 days after infection (Mean± SD). AM, alveolar macrophages. Mo, monocytes. DC, dendritic cells. Neu, neutrophils. (**=P<0.01, ****=P<0.0001, unpaired student's t-test). The experiment was repeated twice with similar results.

The next question asked which APC subsets harbors bacteria during the pulmonary infection *F. tularensis.* The bacterial count in the sorted APC subsets was assessed on 3 or 5 days after intranasal infection (Figure 18). Among the four subsets examined, it was revealed that alveolar macrophages had the highest bacterial count (per 1000 cells), despite their low percentage after infection. This observation is consistent with published data, showing *F. tularensis* mainly replicate in macrophages [140, 183, 187]. Neutrophils, monocytes and dendritic cells also contained live *F. tularensis* LVS, but their burdens of bacteria were not statistically significantly different to alveolar macrophages. Moreover, from day 3 to day 5 after infection, a decreased bacterial intracellular load was observed in all cell subsets which suggested the kinetics of *F. tularensis* LVS infection course in the lungs.

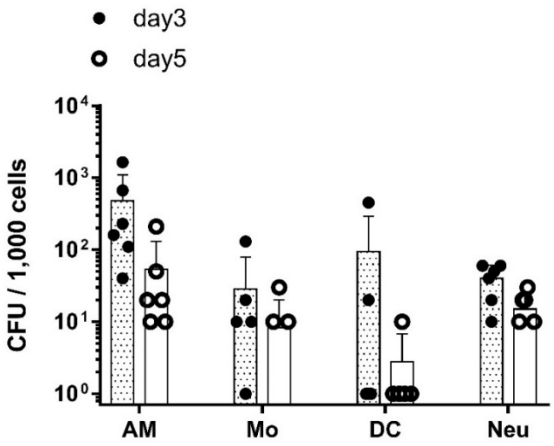


Figure 18 Bacterial load varied in different APC subsets after *F. tularensis* LVS lung infection.

Rag2$^{-/-}$ mice were infected with 150 CFU *F. tularensis* LVS intranasally. Bacterial load of lung APC subsets were determined on 3 or 5 days post-infection by applying 1,000 sorted cells to *F. tularensis* culture plates. AM, alveolar macrophage. Mo, monocyte. DC, dendritic cell. Neu, neutrophil. Bacterial load was presented as CFU per 1,000 cells, Mean±SD. Each dot represents one experimental mouse, and dots that fall at 10^0 represent bacterial loads under the detection limit (less than 10 bacteria per 1,000 cells). No significance, unpaired t-test. The experiment was repeated twice with similar results.

Taken together, these results suggested that APC subsets were infected with *F. tularensis* LVS after the bacteria entered the lungs. During the early phase of *F. tularensis* LVS infection, neutrophils became the dominant population in percentage, while alveolar macrophages had the highest bacterial burden. Our data demonstrated the uptake of *F. tularensis* by each APC subsets, suggesting their potential to present antigen to and activate MAIT cells. The contribution of MAIT cell activation by the *F. tularensis* burdened APC subsets was examined via an *ex vivo* activation assay and is presented in the following sections of the chapter.

4.2.2 Trouble shooting for ex vivo MAIT cell activation assay.

In the previous chapter, an *in vitro* assay with human C1R.MR1 (APCs) and reporter Jurkat.MAIT cell lines was used for detecting antigens for MAIT cells. Similarly, this study seeks to set up an equivalent *ex vivo* assay using sorted murine APCs and MAIT cells. Given that MAIT cell frequencies are low in naïve C57BL/6 mice, boosting MAIT cell numbers *in vivo* facilitates the sorting of sufficient MAIT cell numbers for the assays. Systemic boosting was achieved by intravenous infection of 10^4 CFU *F. tularensis* LVS, and lung MAIT cells were sorted after 1 month, by which time the bacteria were expected to be cleared below the limit of detection but the number and percentage of MAIT cells still remain high (unpublished data from lab members Mai Shi and Zhe Zhao). As the mouse *ex vivo* assay would resemble the human assay in using CD69 upregulation as a readout for assessment of MAIT cell activation, the CD69 expression background of those boosted MAIT cells was examined first.

The results showed that the boosted MAIT cells, although significantly expanded from 0.4-2% to over 20% in the lungs, have an elevated CD69 expression compared to naïve MAIT cells (Figure 19A). In naïve mice, lung MAIT cells express CD69 at very low levels and only ~ 25% of the MAIT cells were $CD69^+$ (Figure 9A). While in donor mice in which MAIT cells had been boosted with *F. tularensis* LVS infection, a high-level expression of CD69 was observed in MAIT cells, in which ~70% of the cells were defined as $CD69^+$ (Figure 9A). The high-level expression of CD69 was maintained in boosted MAIT cells for over 6 months, which become problematic for our *ex vivo* MAIT cell activation assay.

To circumvent this issue, two different approaches were tested. One approach was to sort solely the $CD69^-$ population from *F. tularensis* LVS boosted MAIT cells (Figure 19A). The other possible solution is to use another bacterium for boosting, whose

infection can expand MAIT cells population but without upregulated CD69 expression in harvested MAIT cells. *L. longbeachae* was chosen as a candidate due to accessibility and technical expertise of the host lab. The bacteria were introduced intranasally to boost MAIT cells locally in the lungs. Mice infected with *L. longbeachae* or *F. tularensis* were killed one month after infection, and the CD69 expression of MAIT cells was assessed in the lung and compared to that of MAIT cells from naïve mice. It can be seen from Figure 19B that MAIT cells boosted with *L. longbeachae* resemble naïve MAIT cells in CD69 expression, while *F. tularensis* LVS boosted MAIT cells expressed a significantly higher level of CD69.

To summarize, the boosting strategy influenced the CD69 expression level of boosted MAIT cells. While both of the bacterial strains expanded the MAIT cell population *in vivo* upon infection, at one month after infection *L. longbeachae* boosted MAIT cells showed lower CD69 expression resembling naïve MAIT cells, *F. tularensis* LVS infection induced elevation of CD69 expression in MAIT cells. In order to reduce the CD69 background of MAIT cells to be used in the assay, $CD69^-$ MAIT cells were sorted either directly after *L. longbeachae* boosting, or separately away from the $CD69^+$ population after *F. tularensis* LVS boosting. These $CD69^-$ MAIT cells were used for the *ex vivo* cellular assay thereafter.

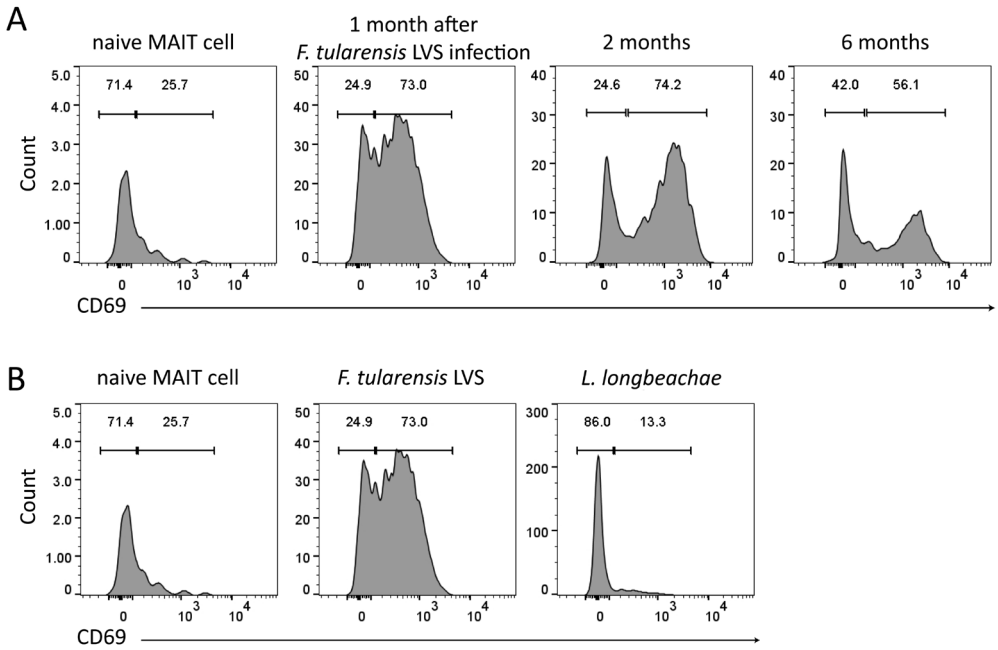


Figure 19 MAIT cells boosted *in vivo* by *F. tularensis* LVS or *L. longbeachae* differ in CD69 expression.

A) MAIT cells were boosted in C57BL/6 mice by intravenous inoculation with 10^4 CFU *F. tularensis* LVS. CD69 expression on MAIT cells was examined before and 1, 2 and 6 months after infection by flow cytometry. Numbers represent percentage of MAIT cells.

B) MAIT cells were boosted in C57BL/6 mice with two strategies. One is intravenous infection of 10^4 CFU *F. tularensis* LVS, and the other is intranasal infection of 10^4 CFU *L. longbeachae.* CD69 expression on MAIT cells was examined 1 month after boosting by flow cytometry. Numbers represent percentage of MAIT cells.

Both experiments were performed twice with similar results.

4.2.3 APCs, especially alveolar macrohages, activate MAIT cells in an MR1 dependent manner when burdened with F. tularensis LVS.

As shown by previous research [140] and this study (Figure 19), MAIT cells are activated, secreting cytokines and expressing early activation maker CD69 after pulmonary *F. tularensis* LVS infection. To examine which cell subset contributes to this activation of MAIT cells, an *ex vivo* co-culture system was developed by incubating MAIT cells with APCs sorted from *F. tularensis* LVS infected mice. Utilizing this system, the MAIT cell activation induced by different subsets of APCs was assessed (Figure 20).

To harvest cells for the co-culture assay, MAIT cells and APCs were sorted from donor mice by flow cytometry as described in the previous section (Figure 3, 16). MAIT cells were first expanded in donor C57BL/6 mice *in vivo* before sorting MAIT cells from lungs. The expansion of MAIT cells was achieved by *F. tularensis* LVS intravenous infection. Infected *Rag2*$^{-/-}$ and control *Rag2*$^{-/-}$*Mr1*$^{-/-}$ mice were chosen for the sources of APCs to allow large numbers of cells to be sorted without T or B cells. APCs were sorted from the lungs on day 3 after intranasal infection with 150 CFU of *F. tularensis* LVS, by which time the APCs were expected to have a high bacterial burden (Figure 18).

With the MAIT cells and APCs prepared, an *ex vivo* co-culture assay was performed (Figure 20). The sorted subsets of APC, including alveolar macrophages, CD103^{+} dendritic cells, neutrophils and monocytes, were co-cultured, separately, with MAIT cells. MR1 ligands (5-OP-RU and 6-FP) and MR1 monoclonal antibody were added to cell culture in some wells for experimental controls. After 5 hours of co-incubation, the activation level of MAIT cells was determined by their CD69 surface expression and assessed by flow cytometry analysis.

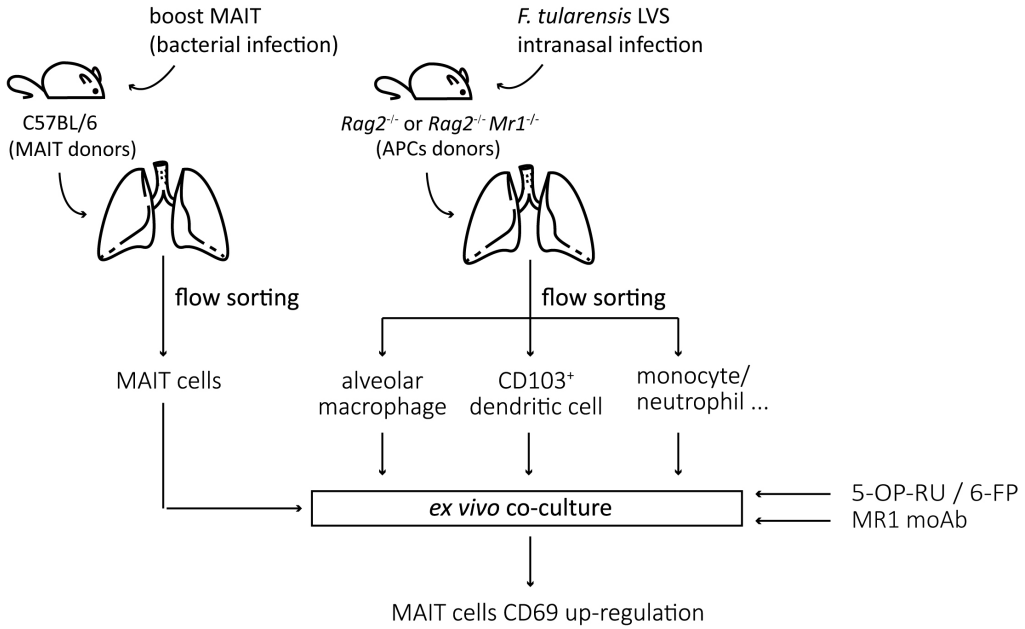


Figure 20 Schematic of *ex vivo* system assaying MAIT cell activation by *F. tularensis* LVS infected APCs.

As MAIT cell donors, C57BL/6 mice were infected with bacteria to boost MAIT cells 6 months before cell harvest. The boosting strategy was either intravenous infection of 10,000 CFU *F. tularensis* LVS or intranasal infection of 10,000 CFU *L. longbeachae*. As donors of APCs, *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Mr1*$^{-/-}$ mice were infected with 150 CFU *F. tularensis* LVS intranasally 3 days before cell harvest. MAIT cells and four subsets of APCs were sorted from mice by flow cytometry, and MAIT cells were co-cultured separately with different APCs for 5 hours *ex vivo*. MR1 ligands (5-OP-RU and 6-FP) and MR1 monoclonal antibody were added to the co-culture in some wells for experimental controls. After incubation, MAIT cell activation was assessed by flow cytometry analysis of CD69 expression.

As investigated earlier in this study, when boosted by *F. tularensis* LVS infection, MAIT cells were driven into two populations, which are $CD69^+$ and $CD69^-$ MAIT cells (Figure 19). By performing two separate experiments with the two MAIT populations, it was first found that, after co-culture with APCs isolated from *F. tularensis* LVS infected mice, a significant increase of CD69 was detected in assays using the sorted $CD69^-$ MAIT cells, but not in $CD69^+$ MAIT populations (Figure 21, 22). In particular, infected alveolar macrophages contributed to the most significant activation of MAIT cells. This activation was abolished when the macrophages lacked MR1 expression (sorted from $Rag2^{-/-}Mr1^{-/-}$ mice) (Figure 22). This result could possibly be explained by the high bacterial load of alveolar macrophages, as has been demonstrated earlier in this study (Figure 18), therefore potentially more MAIT antigens were presented. Apart from alveolar macrophages, monocytes also activated MAIT cells *ex vivo*, but to a relatively lower level comparing to alveolar macrophages.

Taken together, these results suggest that *F. tularensis* infected APCs, especially alveolar macrophages, were capable of delivering ligand to MAIT cells *ex vivo* in an MR1 dependent manner.

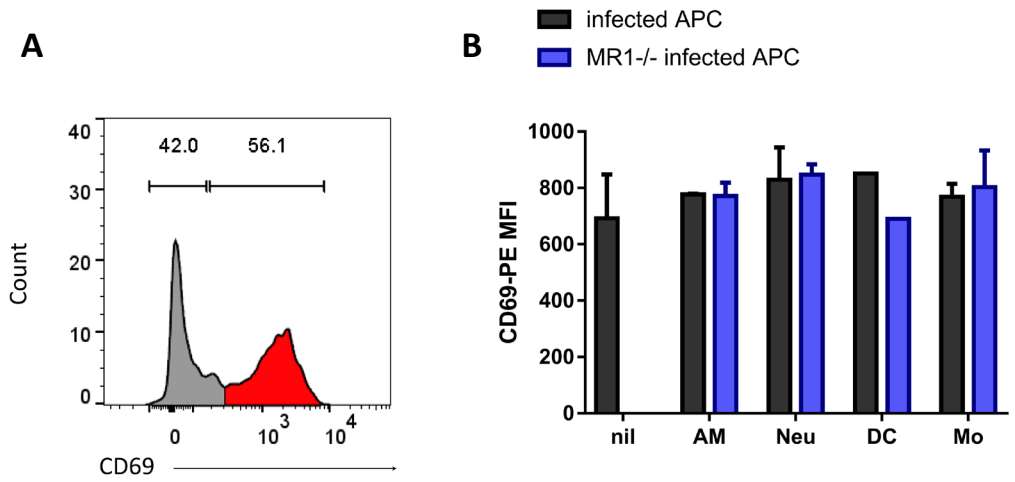


Figure 21 After boosting *in vivo*, CD69$^+$ MAIT cells did not further upregulate CD69 expression following *ex vivo* stimulation.

MAIT cells were boosted by 10^4 CFU *F. tularensis* LVS intravenous infection in C57BL/6 mice and sorted after 6 months. A) CD69 expression of boosted MAIT cells. B) CD69$^+$ MAIT cells, as indicated in red in A), were co-cultured with different subsets of infected APCs from either *Rag2*$^{-/-}$*Mr1*$^{+/+}$ (black) or *Rag2*$^{-/-}$*Mr1*$^{-/-}$ (blue) mice for 5 hours at 37°C. The CD69 expression level of MAIT cells was assessed after incubation. Data showed CD69 expression of MAIT cells in mean fluorescence intensity, MFI (Mean + SD). AM, alveolar macrophage. Neu, neutrophil. DC, dendritic cell. Mo, monocyte. The experiment was performed with duplicate wells and repeated twice with similar results.

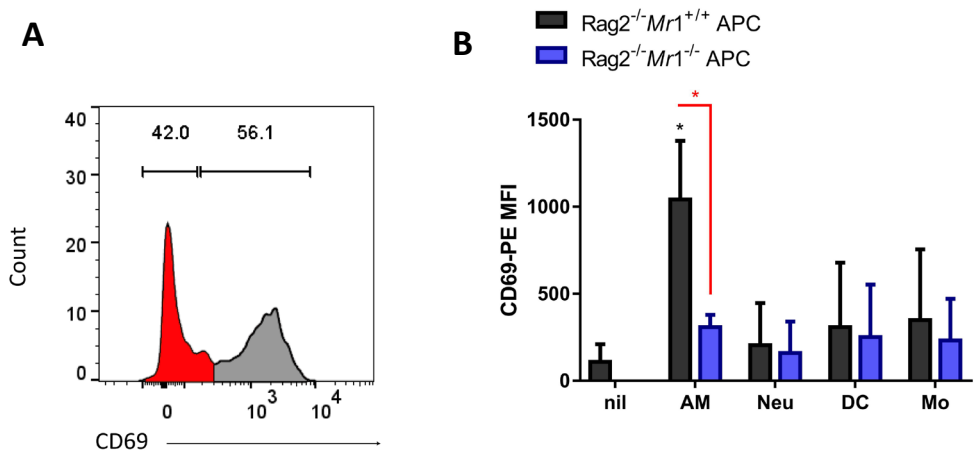


Figure 22 CD69⁻ MAIT cells were activated after co-cultured with lung APCs from *F. tularensis* LVS infected mice.

MAIT cells were boosted by 10^4 CFU *F. tularensis* LVS intravenous infection in C57BL/6 mice and sorted after 6 months. A) CD69 expression of boosted MAIT cells is presented in the left figure. B) CD69$^-$ MAIT cells, as indicated in red in A), were co-cultured with different subsets of infected APCs from both *Rag2*$^{-/-}$*Mr1*$^{+/+}$ (black) and *Rag2*$^{-/-}$*Mr1*$^{-/-}$ (blue) mice for 5 hours at 37°C with 5% CO_2. The CD69 expression level of MAIT cells was assessed after incubation. Data showed CD69 expression of MAIT cells in mean fluorescence intensity, MFI (Mean + SD). AM, alveolar macrophage. Neu, neutrophil. DC, dendritic cell. Mo, monocyte. *=P<0.05, unpaired t-test. The experiment was performed in duplicate wells and repeated twice with similar results. The black asterisk indicates the result of statistical analyse comparing the indicated group with the control group (the first column), and the red asterisk compare the two groups indicated with the red line.

4.2.4 MAIT cell activation by AM is blockable by anti-MR1 antibodies

To further confirm the MR1 dependency of the MAIT cell activation, another *ex vivo* co-culture assay was performed with the presence of anti-MR1 monoclonal antibody (clone: 8F2.F9). In accordance with previous results, both alveolar macrophages (AMs) and equal number of non-AM APCs (a mixture of monocytes, $CD103^+$ DCs and neutrophils) from *F. tularensis* LVS infected $Rag2^{-/-}$ mice, were able to activate MAIT cells (Figure 23). Moreover, the CD69 expression level on MAIT cells significantly lower with the presence of MR1 monoclonal antibody, suggesting that the way MAIT cells respond to the antigen presentation of *F. tularensis* LVS infected APCs is MR1 dependent. This result is similar to what was observed in MAIT cells co-cultured with APCs from $Rag2^{-/-}$ and $Rag2^{-/-}Mr1^{-/-}$ donors, indicating the consistency of the two data sets.

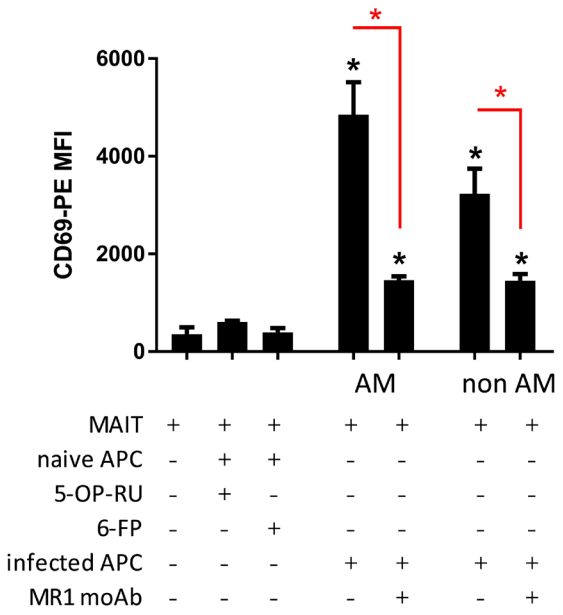


Figure 23 Lung APCs from *F. tularensis* LVS infected mice activated MAIT cells *ex vivo* in an MR1-dependent manner.

C57BL/6 mice were infected with 10,000 CFU *L. longbeachae* intranasally to expand MAIT cell population. *Rag2*$^{-/-}$ mice were infected with 150 CFU *F. tularensis* LVS intranasally and lung APCs were sorted on 3 days post infection. MAIT cells were sorted and co-cultured with either naive or infected APCs for 5 hours at 37°C with 5% CO_2 *ex vivo*. The composition of the cultured mixture is indicated in the table beneath the figure. The CD69 expression level of MAIT cells was assessed after incubation. Naive APCs (AM), 5-OP-RU or 6-FP were added to the separate wells as control groups. Data showed CD69 expression of MAIT cells in mean fluorescence intensity, MFI (Mean + SD). n=4; pooled from two independent experiments. AM, alveolar macrophage. moAb, monoclonal antibody. *=P<0.05, paired t-test. The black asterisks indicate the results of statistical analyses comparing the indicated groups with the control group (the first column), and red asterisks compare the two groups indicated with red lines.

4.3 Discussion

Following a similar principle to a previously established *in vitro* activation assay [4, 5], this study established an *ex vivo* equivalent with sorted murine cells. This model facilitates investigation of MAIT cell activation during an *in vivo* bacterial infection. Specifically, this study focused on the MAIT cell responses in the scenario of *Francisella tularensis* pulmonary infection and found that the activation of MAIT cell was driven by APCs, predominantly by alveolar macrophages (AMs), in an MR1 dependent manner.

F. tularensis is a pathogenic bacterium to both humans and mice, causing severe tularemia pneumonia when inhaled to the respiratory tract. Previous studies have suggested that *F. tularensis* can infect a wide range of lung cells, including macrophages, dendritic cells, neutrophils, monocytes and alveolar epithelial cells, but does not readily invade bronchial epithelial cells [188-191]. However, it is believed that *F. tularensis* primarily infects human and rodent macrophages, including alveolar macrophages and Kupffer cells [183, 192]. Consistent with the literature, the present result found four types of professional APCs all burdened with *F. tularensis* upon pulmonary infection, among which alveolar macrophage had the highest intracellular load of bacteria (Figure 18). Moreover, the bacterial load of APCs decreased from day 3 to day 5 after infection, while the literature suggests that the total bacterial load in the lungs is increasing during this period [140]. This inconsistency may be due to the reproduction of bacteria in other places other than the four APCs assessed in this study. Given the fact that *F. tularensis* is a facultative intracellular pathogen, several phagocytic cells such as interstitial macrophages should be considered as alternative sites for bacterial replication and could be included in any further research [193].

MAIT cells have been indicated to play an important role in efficiently controlling *F. tularensis* pneumonia in mouse experiments [140]. They accumulate locally in the early infection phase and are activated, becoming prominent producers of antibacterial cytokines IFN-γ, IL-17α and GM-CSF [140, 194]. This study suggests that the activation of MAIT cells was contributed by APCs. The APCs have shown to internalize *F. tularensis* upon pulmonary infection (Figure 18). As a corollary, the APCs could potentially present antigens derived from the internalized bacteria. This study helps to unravel the antigen presentation conducted by professional APCs. Burdened with intracellular *F. tularensis*, APCs, especially alveolar macrophages, activated MAIT cells after co-cultured *ex vivo*, and the activation was abolished by

either internal or external depletion of MR1 (Figure 22, 23). These *ex vivo* results reflected those of Meierovics *et al*. (2013) who reported an MR1-dependent activation of MAIT cells induced by bone marrow-derived macrophages infected with *F. tularensis, in vitro* [140]. Unexpectedly, the MAIT cell responses were not completely restrained by anti-MR1 monoclonal antibody (Figure 23), which suggests a synergetic TCR independent, or cytokine dependent manner in triggering MAIT cell stimulation. An isotype control of anti-MR1 monoclonal antibody should also be included ultimately to confirm this finding. The cytokine production of APCs has been indicated by *in vivo* and *in vitro* experiments, which showed that *F. tularensis* appears to replicates in macrophages after internalization, stimulating various degrees of cytokine production in host cells [192, 195]. Moreover, MAIT cells can be activated by cytokines (i.e. IL-12, IL-18) alone [150, 196]. In the circumstance of *F. tularensis* infection, IL-18 was reported to be generated from *Francisella* infected macrophages and worked as a critical stimulus of MAIT cell responses *in vitro* but surprisingly, not *in vivo* [197]. Therefore, how TCR dependent and independent mechanisms function together upon *in vivo F. tularensis* infection remains further exploration.

When optimizing the *ex vivo* cellular assay, some interesting findings about MAIT cell responses were also presented in this study. One observation is that different bacterial infections (with *L. longbeachae* or *F. tularensis*) resulted in different CD69 expression on MAIT cells (Figure 19). One possible explanation is that bacteria produced different amount of ligand that activates MAIT cells, while another reason is that MAIT cells were driven to different phenotypes. It has been reported that MAIT cells were driven to MAIT-1/17 phenotypes and produced IFN-γ and GM-CSF after infection of *L. longbeachae* [101]. While after infection of *F. tularensis*, MAIT cells produced IL-17α and IFN-γ [194], suggesting a different phenotype of MAIT cells from that after *L. longbeachae* infection. Given that distinctive expression level of CD69 has been reported between two functional phenotypes of MAIT cells [164], it is reasonable to speculate that MAIT cells were driven to different phenotypes after infection, which resultantly influenced CD69 expression.

Another observation is that $CD69^+$ MAIT cells could not further upregulate their CD69 expression by co-culture with infected APCs, which is within expectation (Figure 21). $CD69^-$ MAIT cells still showed sensibility to activation signals provided by the infected APCs (Figure 22), but surprisingly, these MAIT cells could not be stimulated efficiently by MAIT cell ligand 5-OP-RU with naïve APCs, which was expected as a positive control of *ex vivo* assay (Figure 23). One possible explanation might be the extremely low MR1 expression on murine APCs at steady states [104,

198]. This explanation was not supported by *in vitro* studies with human cell lines, which showed the MR1 expression could be elevated with the presence of cognate ligand [4, 5, 130, 199, 200]. However, there are still possibilities that *in vitro* studies using human cell lines may not always reflect the physiological events (*in vivo* or indirect *ex vivo* findings) in mice. Therefore, further research should be undertaken to investigate if other elements including danger stimuli (i.e. TLR agonists) are required for APC-induced MAIT cell activation *in vivo* and *ex vivo*.

Overall, this study established an *ex vivo* assay and showed that various APCs, especially alveolar macrophages, are important in initiating a MAIT cell response during *F. tularensis* lung infection. The findings will contribute to understanding the host immunity against *F. tularensis*, and additionally, provide a new and practical system to assay MAIT cell behaviours in other infection or non-infectious diseases.

Chapter 5: General Discussion

MAIT cells comprise a relatively large proportion of T cells in humans or a smaller proportion in mice and display tropism to mucosal tissues. MAIT cells are MR1-restricted and recognize vitamin B2 (riboflavin) metabolites, which are conserved products of microbial metabolism but are not produced by mammals. Once they detect antigens, MAIT cells can rapidly respond and protect the host against microbial infections, as shown by a variety of *in vitro, in vivo* and *ex vivo* studies. However, *in vitro* observations do not always reflect *in vivo* activities of MAIT cells [197], and *in vivo* and *ex vivo* research methods and systems for studying MAIT cells are to date still limited. Aimed at unravelling the MAIT cell responses during bacterial infection, this study developed two novel systems which would facilitate clinical and laboratory MAIT cell studies. In Chapter 3, *in vivo* mouse infection models were established to mimic bacterial peritonitis in human patients, and MAIT cell activation and protective roles were demonstrated in a few aspects. In Chapter 4, an *ex vivo* cellular assay was optimized, in which the initiation of MAIT cell activation induced by antigen presenting cells during *in vivo* infection was explored. In the following sections of Chapter 5, the perspectives and limitations of the current study are discussed.

5.1 Peritonitis as an infection model for MAIT cell studies

MAIT cells and their antibacterial properties have been demonstrated by a variety of *in vitro* or *in vivo* studies, either with human cells or in animals. With wildtype or transgenic mice which harbour more MAIT cells, several *in vivo* models have been developed in which the vital importance of MAIT cells in controlling infection was highlighted (Table 2). However, only in pulmonary infections have the MAIT cells been investigated extensively (e.g. with *L. longbeachae*, *Mycobacterium spp.* and *F. tularensis* infections) [101, 140, 142, 144]. Upon infections of other sites such as the peritoneal cavity, protective roles were suggested to be played by MAIT cells but still remained equivocal [75, 143]. This study established *in vivo* bacterial peritonitis models and for the first time, demonstrated that these models are functionally applicable for investigating MAIT cell responses.

Bacterial peritonitis models are not novel to microbiology or immunology studies but are just starting to emerge in the field of MAIT cell research [201, 202]. Previous

comparison studies showed augmented morbidity in *Mr1*$^{-/-}$ mice during *E. coli* and *K. pneumoniae* induced peritonitis [75, 143]. However, the mechanisms of MAIT cell protection particularly in the peritoneal cavity remained unexamined. Results from this dissertation suggest peritonitis is a valuable infection model for studying MAIT cell responses. Upon *E. coli* induced peritonitis, MAIT cells amassed in the peritoneal cavity as well as multiple organs, reaching ~5% αβ T cells in the cavity and ~20% in the liver, which was a significant increase compared to their percentage in naïve mice (~0.3% in the cavity and ~0.5% in the liver) (Figure 11, 12). In accordance with present results, two independent human studies performed by Le Bouhris *et al.* and Jiang *et al.* demonstrated that MAIT cell amassed in the peritoneal cavity in patients with *M. tuberculosis* ascites compared to that of malignant ascites [75, 203]. By contrast, MAIT cell accumulation was weak in other body fluid cavities affected by *M. tuberculosis*. In patients with tuberculosis meningitis or pulmonary tuberculosis, MAIT cell percentage remained low in cerebrospinal fluid (1% αβ T cells) and in pleural effusions (0.91%) respectively [203-205]. Moreover, in both wild type mice and rhesus macaques models of pulmonary tuberculosis, mild and nonsignificant changes of MAIT cell number in bronchoalveolar lavage fluid (BALF) before and after infection was reported, suggesting that pulmonary infection and BALF analysis were potentially less representative and compelling models in studying MAIT cell responses against tuberculosis [141, 206]. Therefore, the peritonitis models proposed by this dissertation could become an alternative option and facilitate MAIT cell research.

The importance of the present study is also underlined by the significance of treating bacterial peritonitis in the clinic. The peritoneal cavity is the largest serosal cavity in humans, containing serous fluid and immune cells. Failure in controlling bacterial invasion into peritoneal cavity and peritoneum induce bacterial peritonitis, which accounts for 30% to 50% of the mortality in liver disease patients and with a mortality rate ranging from 20% to 80% even with antibiotic treatments [154, 207]. In this study, MAIT cells were found to control bacterial growth in peritoneal cavity and reduce death rate in mice with *E. coli* peritonitis (Figure 13), which indicates that MAIT cells are important components in defending against bacterial peritonitis in mice and, potentially, in human patients as well. Admittedly, the present study has not deeply investigated how important are MAIT cells in controlling bacterial peritonitis. Further *in vivo* experiments comparing the outcome of infected mice with depletion of either MAIT cells or other types of immune cells would be needed to make a more solid statement.

Clinical studies have demonstrated that a well-orchestrated immune response is required for successful control of bacterial peritonitis, including phagocytosis of peritoneal macrophages that constructed the first line defence, infiltration of leukocyte and subsequently mobilized adaptive immunity [208]. But which exact part do MAIT cells play? The present study showed that MAIT cells were strongly polarized to Th1 phenotype (T-bet^{+}) and produced IFN-γ (Figure 14, 15), a potent macrophage stimulatory factor that enhance phagocytosis [171, 172]. Given that peritoneal macrophages and polymorphonuclear leukocytes are primary and major immune cells in the defence against bacterial peritonitis [201, 209], it is conceivable to propose that MAIT cells promote the killing activities of phagocytes, and the engulfed bacteria are consequently destroyed [201, 210]. Admittedly, this study was limited in terms of exploring the significance of IFN-γ produced by MAIT cells and other downstream mechanisms of MAIT cell protection against *E. coli* peritonitis. Future research on the current topic is therefore recommended. Nevertheless, the apparent selective increase of T-bet^{+} MAIT cells in the peritoneal cavity might reveal important information such as the functional plasticity of MAIT cells upon i.p. bacterial infection. These results might have direct implications on the possible choice of experiment systems for future MAIT cell studies *in vivo* as well as the functional analyses of MAIT cell properties.

Overall, this study helped establish a bacterial peritonitis model and in conjugation with previous observations in human patients, suggest a consistent MAIT cell response in the peritoneal cavity. This peritonitis model also supplemented the established *in vivo* models to achieve the variety of MAIT cell investigation systems, which would be useful to address broader questions about MAIT cell biology such as MAIT cell trafficking and immunological memory. The findings that MAIT cells accumulated, became activated and delivered protection upon bacterial peritonitis are potentially beneficial for either laboratory or clinical investigations.

5.2 *Ex vivo* assay as a new system for unravelling MAIT cell responses *in vivo*

The mechanisms of MAIT cell activation have been demonstrated in accumulating *in vitro* cellular studies which shows that various types of mammalian cell can activate MAIT cells upon bacterial infection [211]. However, the activation process of MAIT cells *in vivo* has not been fully understood. An array of bacteria that possess a

riboflavin biosynthesis pathways and thus are thought to be capable of producing MAIT stimulatory antigens have been shown to induce MAIT cells activation *in vitro* but not necessarily *in vivo* [197, 212]. This has been observed in this study as well, as three bacteria, *S. aureus*, *K. pneumoniae* and *E. coli*, all stimulated MAIT *in vitro* but only *E. coli* induced significant MAIT cell responses *in vivo* (Figure 5, 12). There could be many reasons for the observed inconsistency between *in vitro* and *in vivo* data sets, one of which is that *in vivo* immune responses are much more complicated as they are orchestrated with many facets of immune system.

To achieve a better understanding of MAIT cell responses in a "natural" scenario of infection, an *ex vivo* assay was developed with MAIT cells and APCs in this study, which is similar to that of published *in vitro* assays [4, 5, 75]. Utilizing sorted MAIT cells from human PBMC or from Vα19*i*Tg mice, Gold *et al.* and Le Bourhis *et al.* have first demonstrated that bacterially infected APCs could stimulate co-cultured MAIT cells *in vitro* [75, 109]. This *in vitro* assay has been extensively developed in studies thereafter, in which researchers reported that MAIT cells were activated when cultured with *E. coli* infected THP-1 cells (a human monocytic cell line) [116], *M. tuberculosis* infected DCs or epithelial cells [130], or suppressed the intracellular growth of *M. bovis* BCG in bone marrow-derived macrophages [114]. However, co-culture of peripheral MAIT cells with non-peripheral APCs or APC cell lines cannot reflect an actual interaction of immune cells during *in vivo* immune responses. Therefore, the previous *in vitro* experiments were focused on understanding the molecular basis of MAIT cell activation and activities rather than *in vivo* immune responses of MAIT cells upon infections. Direct stimulation of PBMC with bacteria was an alternative assay that mimicked bacterial sepsis in clinic, and a variety of recent studies have shown that upon bacterial stimulation, MAIT cells became activated with a complete or incomplete dependency of MR1 [116, 130, 203]. But these assays have been restricted to the immune responses in the blood where infections are as common as in mucosal tissues. In the *ex vivo* assay presented in this dissertation, MAIT cell responses were investigated in the circumstances of pulmonary infections, which are more frequently than bacterial sepsis in clinic. With this aim, both MAIT cells and APCs that used for the *ex vivo* co-culture assay were sorted from lung tissues. Notably, different from previous assays in which the cells were infected *in vitro*, the present *ex vivo* assay was performed by culturing sorted cells from infected mice. These cells underwent an *in vivo* course of bacterial infection and thus, the *ex vivo* assay better reflected *in vivo* cellular infection processes and interactions.

Utilizing the established *ex vivo* assay, this study further investigated the activation process of MAIT cells during *F. tularensis* pulmonary infection. Infected antigen presenting cells, particularly alveolar macrophages, stimulated MAIT cells in an MR1 dependent manner (Figure 22, 23). The *ex vivo* stimulatory results were in consistent with the *in vivo* stimulation of MAIT cell upon *F. tularensis* infection reported by Meierovics *et al* [140], suggesting that the *ex vivo* system might reflect *in vivo* responses. Therefore, the *ex vivo* assay developed from this dissertation provides a novel tool for MAIT cell research by mimicking immune responses *in vivo*. The major limitation of this study is that only *F. tularensis* infection was examined, and thus more bacterial infection process could be assessed with this *ex vivo* system in future analysis of MAIT cells.

5.3 Conclusion of the thesis

To conclude, this study explored the antibacterial properties of MAIT cells with two newly developed models. Firstly, with established bacterial peritonitis models in mice, this study demonstrated that a robust *in vivo* expansion and activation of MAIT cells were induced in peritoneal cavity and in multiple organs upon *E. coli* peritonitis. Secondly, this study optimized an *ex vivo* cellular assay in which the activation of MAIT cells was found to be stimulated by APCs, especially alveolar macrophages, in an MR1 dependent manner during *F. tularensis* infection. The two models have provided new useful tools for further research for a more comprehensive understanding of the immune responses and antibacterial properties of MAIT cells. Also, findings of this dissertation suggest MAIT cells as potential therapeutic targets for clinical infectious diseases.

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