Mucosal-associated invariant T cells and *Aspergillus fumigatus* infection

Thesis submitted in total fulfilment of the requirements of the degree of Master of Research

Bingjie Wang

Supervisors: Zhenjun Chen, Alexandra Jane Corbett, James McCluskey

Faculty of Medicine, Dentistry and Health Science
The University of Melbourne

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ORCID: 0000-0003-3654-6227

Abstract

Mucosal-associated invariant T (MAIT) cells are an abundant population of T cells, especially in humans, that express a conserved αβ T cell antigen receptor (TCR), which is restricted to a highly conserved monomorphic MHC class I -like related molecule (MR1). These high frequencies and high conservation suggest their importance in the immune system. MAIT cells can be activated by vitamin B metabolites derived from riboflavin synthetic pathway. This pathway is conserved in bacteria and fungi, which gives MAIT cells the opportunity to recognize these pathogens and participate in host defense.

Aspergillus *spp.* are common saprophytic fungi, which grow on decaying organic matter and normally do not cause disease in healthy individuals. However, some species, including *Aspergillus fumigatus* (*A. fumigatus*), can be pathogenic and can cause disease in people with chronic lung diseases or immunodeficiencies. With the emergence of multi-drug resistant Aspergillus strains, there is a need for better understanding of the immune defense against Aspergillus in order to develop new therapeutic strategies. Aspergillus *spp.* can synthesize riboflavin and it was recently found that Aspergillus conidia can stimulate human MAIT cells *in vitro*. Here, a mouse model was established with *A. fumigatus* pulmonary infection to assess MAIT cell responses *in vivo*. Results presented in this dissertation demonstrate a robust accumulation of pulmonary MAIT cells in infected mice, with a Th17-like phenotype and large IL-17A production in the early stage. The strong response of MAIT cells to infection suggests their role in host resistance against *A. fumigatus* infection. Fungal burden was compared between WT mice and *Mr1*$^{-/-}$ mice, and immunocompromised mouse models were used to investigate differences in fungal burden in the presence or absence of MAIT cells. Although further optimization will be needed, these models provide a basic understanding of MAIT cell responses during pulmonary infection with *Aspergillus fumigatus*, including the dose response and kinetics.

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: 23/07/2020

Bingjie Wang

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Abbreviations

5-A-RU	5-amino-6-D-ribitylaminouracil
5-OE-RU	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
6-FP	6-formylpterin
7-AAD	7- Aminoactinomicin D
ABPA	Allergic bronchopulmonary aspergillosis
Ac-6-FP	Acetyl-6-formylpterin
APC	Antigen presenting cell
BCG	*Mycobacterium bovis* Bacillus Calmette-Guérin
BCR	B cell receptor
CA	Cortisone Acetate
CBA	Cytometric bead array
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CFU	Colony-forming unit
CLR	C-type lectin receptor
CTX	Cyclophosphamide
DC	Dendritic cells
DN	Double negative
DP	Double positive
DPI	Days post infection
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FOXP3 (Foxp3)	Forkhead box P3
FVD	Fixable Viability Dyes
GC	germinal centers
GEM-T	Germline-encoded mycoly lipid-reactive T
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMS	Grocott's methenamine silver
HE	Hematoxylin and Eosin

HLA	Human leukocyte antigen
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
i.v.	Intravenous
ICOS	Inducible T-cell costimulator
ICS	Intracellular cytokine staining
IFN	Interferon
Ig	Immunoglobulins
IL	Interleukin
ILCs	Innate lymphoid cells
LAP	LC-3 associated phagocytosis
LN	Lymph node
LPS	Lipopolysaccharides
LVS	Live Vaccine Strain of *Francisella tularensis*
mAb	Monoclonal antibody
MAIT	Mucosal-associated invariant T
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
MR1	MHC class I-like related molecule
NET	Neutrophil extracellular trap
NHEJ	Non-homologous end joining
NK	Natural killer
NKT	Natural killer T
NLR	NOD-like receptor
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
pDC	Plasmacytoid dendritic cell
PLZF	Promyelocytic leukemia zinc finger

PMA	Phorbol 12-myristate 13-acetate
PRR	Pathogen-recognition receptor
RL-6,7-diMe	6,7-dimethyl-8-D-ribityllumazine
RL-6-Me-7-OH	7-hydroxy-6-methyl-8-D-ribityllumazine
RLR	RIG-I-like receptor
RORγt	RAR-related orphan receptor gamma
RPM	Revolution(s) per minute
RPMI	Roswell Park Memorial Institute
s.c.	Subcutaneous
S/N	Supernatant
SA	Streptavidin
SAFS	Severe asthma with fungal sensitization
SC	Supplementary components
SEM	Standard Error of the Mean
SPF	Specific pathogen-free
Spp.	species
TAC	Tris-buffered ammonium chloride
TCR	T cell receptor
TF	Transcription factor
Tfh	T follicular helper
Tg	TCR-transgenic
Th	T helpter
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	regulatory T
WT	Wild type
β_2m	β_2-microglobulin

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Chapter 1 Introduction

1.1 The immune system

The immune system recognizes antigens, including from infectious pathogens and foreign grafts, and confers immune responses to protect the body. An effective immune response needs precise and efficient cooperation of many components, including immune organs, cells and molecules [1]. Defects of the immune system may result in increased susceptibility to infection or uncontrolled response to antigens which could lead to autoimmune diseases (e.g. systemic lupus erythematosus) or tissue damage (e.g. death by cytokine storm). Classical immunity includes mainly two categories: innate immune system and adaptive immune system [1].

1.1.1 Innate immune system

The innate immune system separates or removes pathogens in most cases, especially for organisms without an adaptive immune arm. It includes anatomical barriers (such as skin, mucosa, barriers like blood-brain barrier) [2], innate immune cells including natural killer (NK) cells, mast cells, eosinophils, basophils and the phagocytic cells such as macrophages, neutrophils and dendritic cells (DC) [3], and innate immune molecules (such as complement proteins, chemokines, cytokines). The physical barriers including the skin and mucus stop most pathogens from entering the body [2]. Under some conditions, when pathogens succeed in breaching physical barriers, other innate immune components respond very rapidly. Antimicrobial enzymes can lyse bacteria directly and the complement system will lyse antibody-opsonized pathogens and trigger the recruitment of inflammatory cells [4, 5]. These effector cells recognize pathogen-associated molecular patterns (PAMPs) which are small molecular motifs including lipopolysaccharides (LPS), bacterial flagellin, and nucleic acids [6, 7]. PAMPs are conserved within a class of microbes and not shared by host cells [8]. This makes the innate immune system able to distinguish non-self-ligands from host ligands, and respond within hours, although non-specifically [9].

The innate immune system recognizes PAMPs via receptors on macrophages, neutrophils and DCs termed pathogen-recognition receptors (PRRs). These include Toll-like

receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) [10-13]. Activation of these receptors can trigger phagocytosis and signaling pathways leading to production of cytokines and interferons to kill infectious agents [14-16]. The expression of cytokines is very important as this helps mobilize other immune cells, and induces and maintains inflammation to "mark" the sites [1].

During inflammation, neutrophils are one of the first cell types attracted to the site. They are usually found in bloodstream and can move freely through blood vessels and interstitial tissue, and attack pathogens immediately. Neutrophils can be identified with surface markers including Myeloperoxidase (MPO), Cluster of differentiation 16 (CD16), CD66 and CD11b in humans or with Ly6G (also expressed by monocytes and granulocytes) in mice. Neutrophils are phagocytes and can ingest pathogens [17]. They also can release soluble anti-microbials by a process called degranulation or generate neutrophil extracellular traps (NETs) to attack pathogens [18]. Neutrophils also have important role in the recruitment of other immune cells, such as dendritic cells (DCs) and macrophages [19]. After ingesting pathogens, neutrophils undergo programmed cell death via a process known as apoptosis [20]. The cell debris will be removed by macrophages [21].

Macrophages are phagocytic cells. They reside in tissues and can be derived from circulating monocytes and recruited at the time of inflammation [22]. They can ingest dying or dead cells, cell debris, and foreign material such as pathogens. After digestion of the pathogen, macrophages present antigen to helper T cells, triggering adaptive immunity [23]. Macrophages can secrete many products which affect the physiology of other cells [24]. For example, interleukin (IL) -1 secreted by macrophages supports the activation of B lymphocytes and lymphokine production of T lymphocytes [25].

Innate lymphoid cells (ILCs) are a group of innate immune cells. They do not express antigen specific B or T cell receptors and respond to infection quickly; but they can secrete cytokines like T lymphocytes. Based on the cytokines they produce, they can be divided into three subsets [26]: Group 1 ILCs, which are able to produce T helper 1 cell (Th1) cytokines including Interferon (IFN) -γ and tumour necrosis factor (TNF); Group

2 ILCs, which produce type 2 cytokines like IL-4, IL-5, IL-9 and IL-13; and Group 3 ILCs, which produce IL-17A and/or IL-22.

NK cells are a subset of group 1 ILCs. They express the surface markers NK1.1 or NK1.2 in C57BL/6 mice, and CD16, CD56 and CD94 in humans [26]. The activation of NK cells depends on the balance of activating and inhibitory receptor stimulation. Inhibitory receptors can recognize major histocompatibility complex (MHC) Class I alleles and prevent killing by NK cells. For cells which have lost MHC Class I markers (called as "missing self"), NK cells are activated and cause lysis of these cells [27]. NK cells can show a cytotoxicity response (such as releasing perforin and granzymes and inducing apoptosis or osmotic cell lysis) and produce IFN-γ in the first few hours to several days after primary infection [28], and thus, they play an important role in response to virus-infected cells [29] and tumor formation [30, 31]. The function of NK cell can be affected by other cell types; the cell-to cell interaction between NK cells and DCs was showed to affect the activation, maturation of both cells types, which enhanced the proliferation and cytotoxicity of NK cell and the maturation of DCs [32].

DCs are the major antigen-presenting cells (APCs) of the immune system. DCs link innate and adaptive immunity and are believed to be the only APCs to efficiently prime naïve T cells in the lymph node (LN) [33, 34]. DCs are able to capture, process and present antigens to T cells, participate in T cell selection, and can migrate to lymph nodes to interact with naïve T cells and B cells [35]. DCs can be divided to two different subsets: conventional dendritic cell (cDC) and plasmacytoid dendritic cell (pDC) [36]. cDCs are good APCs and can capture antigens by phagocytosis [37], micropinocytosis [38], and adsorptive endocytosis [38-40]. Antigens taken by cDCs are processed and assembled as antigen-MHC class I or II complexes and presented to $CD8^+$ or $CD4^+$ T cells respectively in the LN [41]. In the thymus, cDCs present self-antigens to help build T cell tolerance by the deletion of autoreactive T cells [41]. cDCs also can affect immune response by producing cytokines such as IL-12 [42], which is critical for Th1 response [43]. pDCs appear round before activation and acquire a dendritic shape after activation [44], they can detect special structures of viral nucleic acids through TLRs (including TLR-7 and TLR-9) [45] and quickly secrete type I and III IFNs in response to viral infections, such as IFN-γ, which can activate both innate immunity (such as NK cells) and adaptive

immunity (such as B cells) [46]. pDCs also can act as APCs, although they are less efficiently than cDCs because of the low expression of antigen-presenting related molecules such as MHC class II [47]. Activated pDCs can induce expansion of antigen-specific adaptive immune cells such as anti-influenza virus $CD8^+$ and $CD4^+$ T cells populations [48].

Innate immunity reacts quickly when faced with external aggression, it can help with host defense by directly removing pathogens and interacting with adaptive immunity through various signals. If the innate immune system does not successfully remove the pathogen, the adaptive immune system joins in.

1.1.2 Adaptive immune system

Although the innate immune system can act quickly to defend the body, its functionality is limited because of the lack of specificity and limited molecular patterns it can recognize [1, 4]. In addition to acting on pathogens and infected cells, innate immunity can also help in host defense by triggering the activation of the adaptive immune system. DCs and macrophages present antigens to adaptive immune cells and help them mature and activate [23, 35]. Cytokines, such as IL-1, secreted by innate immune cells support the effector functions (such as antibody and lymphokine production) of lymphocytes [25]. The adaptive immune system protects the host from a great variety of pathogens in a more specific way [1, 4]. It consists of two parts: the humoral response, which is mediated by antibodies produced by effector B cells (plasma B cells), and the cell-mediated response, which is mediated by T cells. The specificity of adaptive immunity is achieved by the membrane-bound proteins which serve as antigen receptors on B cells and T cells, known as the B cell receptor (BCR) and T cell receptor (TCR), respectively [49, 50]. Both BCR genes and TCR genes have variable (V), joining (J) and diversity (D) segments [51-53], the recombination and rearrangement of these segments give each B cell or T cell a unique antigen specificity and generates an enormous diversity of TCRs and BCRs to recognize nearly unlimited known or unknown antigens [52].

Antibodies, also known as immunoglobulins (Ig), are the secreted form of BCRs, they are soluble and can directly bind antigens [54]. There are 5 main classes of antibodies, including IgM, IgG, IgA, IgD, and IgE [55]. Antibody proteins are composed of 2 light

(L) chains and 2 heavy (H) chains. Both L chains and H chains contain a variable (V) domain and a constant (C) domain [56]. The variety of V domains are achieved by the rearrangement of gene segments, which make antibodies have the capability to recognize different ligands [57]. Antibodies can block the effective parts on the surface of bacteria or virus, resulting in neutralization, or they can mark, agglutinate or precipitate antigens to make them become targets (a process called opsonization) for phagocytosis, as well as activate the complement system to lyse pathogens and encourage inflammation [55].

The TCRs, in contrast to BCRs, cannot recognize antigens by themselves. They need other host cells to present antigens to them through specific proteins known as MHC molecules [58]. There are many kinds of T cells which function in cell-mediated immunity. Cytotoxic T cells are able to induce apoptosis to destroy infected cells [59], and T helper cells have the ability to activate macrophages or stimulate B cells producing antibodies [60].

Some of the B cells and T cells generated in adaptive immune responses persist for a long time while most others eventually die after the antigen is cleared. These cells, known as memory cells, can provide long-lasting immunological memory and protection against subsequent antigen or infectious challenge [61, 62], which is the foundation of vaccination. Vaccination is the most effective means of controlling infectious diseases, and it has dramatically improved public health worldwide [63].

1.2 T cell immunity

T cells play a central role in the adaptive immune response. They originate from precursor cells which reside in the bone marrow and migrate to the thymus gland via the blood stream [64]. Within the thymus, immature T cells recombine and rearrange their TCR genes and create a functional TCRβ-chain to pair with the invariant pre-TCR α-chain (or TCRγ-chain pair with TCRδ-chain for γδ T cells), forming a pre-TCR. After the rearrangement of α-chain, pre-TCR becomes the full TCR. Those cells that express a full TCR will then undergo positive selection and negative selection, which remove T cells that do not recognize self MHC and that strongly interact with self-antigen [65]. The remaining cells exit the thymus as mature naïve T cells and circulate in the blood and peripheral lymphoid tissues, where, if they encounter specific antigen and further develop

into specialized T cells [66, 67]. According to their surface expression of cluster of differentiation (CD) molecules, conventional T cells are classically divided into CD4+ (helper) and CD8+ (cytotoxic) T cells.

In the thymus, CD4+ T cells and CD8+ T cells interact with different MHC molecules. MHC class I molecules are expressed on all nucleated cells and are recognized by T cells expressing CD8 co-receptors [68]. MHC class II molecules are normally expressed by professional APCs, including DCs, B cells, and macrophages, and can present extracellular antigens to CD4+ T cells [69-71]. MHC molecules are highly polymorphism in vertebrates because of the polygeny and the codominant expression of these genes [72]. In humans, MHC class I and II molecules are also called human leukocyte antigen (HLA). There are at least six different HLA molecules expressed in each individual, and much greater diversity in the population [73]. It was shown that MHC molecules control the antigen specificity of T cells, thus the polymorphism of MHC enlarges the range of antigens that the immune system can recognise [74]. The binding of TCRs and MHC molecules provides the first signal for T cells activation. In addition, T cell activation also needs second signals called co-stimulation, otherwise the first signal alone will result in anergy [75]. Second signal include the co-stimulatory molecules, such as CD28, inducible T-cell costimulator (ICOS), and CD40L on T cells and B7 (CD80 and CD86), ICOSL, and CD40 on APCs [76, 77]. These two signals with certain cytokines activate T cells, initiate proliferation and determine the differentiation directions [78].

1.2.1 Conventional T cell function

“Conventional T cells” refers to peptide-reactive CD4+ and CD8+ T cells that are restricted by classical MHC I or II complexes [79, 80]. Helper CD4+ T cells (T helper cells or Th cells) assist other lymphocytes by producing cytokines [81]. After activation they can differentiate into one of several subtypes, including T helper 1 (Th1), Th2, Th9, Th22, Th17, regulatory T (Treg), and T follicular helper (Tfh) cells, which play dissimilar roles in the immune system [82].

Th1 and Th2 cells were discovered in the late 1980s. Th1 cells respond to signals such as IL-12 [83] and need transcription factors such as STAT4 and T-bet for development [84]. They are functional in controlling intracellular bacteria, viruses and cancer. The principal

effector cytokine of Th1 cells is IFN-γ, which can activate macrophages, stimulate IgG production, enhance Th1 function and inhibit the activity of Th2 cells [60, 85]. Cross-regulation was described between Th1 and Th2 cells, mediated by cytokines produced by each subsets [85, 86]. Th2 type cytokines such as IL-10 and IL-4 were found have the function to block Th1 cells activation [86], and contribute to the differentiation and maturation of B cells [87].

Th9 cells were demonstrated in 2008 which producing mainly IL-9, contribute to immunopathology in autoimmune diseases, anti-tumor immunity, and allergy [88]. Th22 cells primarily secrete IL-22 [89], which has a role in both inflammation and tissue repair [90]. Tfh select B cells in germinal centers (GC) and help with the maturation and proliferation of GC B cells [91].

Th17 cells produce IL-17 and function in infection, autoimmunity, immunodeficiency and neoplastic diseases [92-94]. Treg cells function to restrain excessive T cell response and are crucial for preventing autoimmune diseases and maintaining immunological tolerance [95]. The balance between Th17 and Treg cells was found to be regulated by many factors such as IL-6 and forkhead box P3 (FOXP3) [96, 97].

Cytotoxic CD8+ T cells (T_C cells, CTLs) can directly kill cancer cells or infected cells [98]. T_C cells recognize their targets by the specific antigen presented by MHC class I molecules, which are expressed on all nucleated cells [68]. When activated, T_C cells can release perforin, granzymes and granulysin to trigger apoptosis of target cells [59]. They can also trigger apoptosis via cell-surface receptor interactions (binding of Fas ligand on T_C cells and Fas molecules on target cells) [99]. Most T_C cells also secrete some cytokines, like IFN-γ and TNF, which contribute to anti-microbial and anti-tumor effects [100].

1.2.2 Innate-like T cells

Some T cells are derived from common lymphoid progenitor cells but share some properties of both innate immune cells and conventional T cells. These innate like T cells blur the boundaries between the innate and adaptive immune system. Among them, some cells express TCR molecules but are not restricted to classical MHC molecules [1], such as CD1-Restricted T cells, γδ T cells (unlike the majority of T cells, they express γδ TCR

chains rather than α and β chains), and mucosal associated invariant T cells (MAIT cells). These cells have more limited receptor repertoire and recognize non-peptide antigens compared to conventional T cells, but are also crucial in the immune response during infections and autoimmunity [101].

CD1-Restricted T cells recognize lipid antigens presented by CD1 molecules [102]. CD1 is a family of MHC class I related molecules which expressed on APCs and can present lipid antigens to T cells [103]. CD1-Restricted T cells can be divided to two groups according to the CD1 subsets they restricted [103]: group I CD1-restricted T cells which are restricted to group I CD1 molecules (CD1a, CD1b, and CD1c, expressed in humans and other vertebrates, but not in mice and rats) and CD1d-restricted T cells which also designated Natural Killer T (NK T) cells.

Germline-encoded mycoly lipid-reactive T cells (GEM-T cells) express αβ TCRs and recognize antigens such as mycobacterial mycolates present by CD1b molecule [104]. GEM-T cells expand upon *Mycobacterium tuberculosis* infection and help with host defense through IFN-γ and TNF-α [105].

NKT cells express αβ TCRs and NK cell-associated markers, such as NK1.1, they are restricted by CD1d molecules. NKT cells recognize a large range of different lipid classes, such as glycosylceramides and glycosphingolipid [106]. Activated NKT cells directly kill target cells and/or produce large amounts of cytokines. They can function in anti-microbial infections and anti-tumour immunity [107, 108].

γδ T cells have distinctive TCRs, which consist of γ and δ chains, and are abundant in many tissues. Like α and β chains of conventional TCRs, γ and δ chains undergo recombination and rearrangement to produce junctional diversity. However, the antigen patterns they can recognize are more limited than αβ T cells. Most γδ T cells' restriction and cognate Ag (s) have not been fully understood, except a few cases such as Vγ9Vδ2 TCRs, which are expressed by most circulating human γδ T cells, and specifically react to phosphoantigens and butyrophilins [109]. Activated γδ T cells produce many cytokines during inflammation and tissue repair [110, 111].

1.3 Mucosal associated invariant T (MAIT) cells

MAIT cells are a kind of innate-like T lymphocytes and were first identified in 1999 [112]. They express a conserved αβ TCR, which consists of a semi-invariant TCR α chain (TRAV1-2 (Vα7.2) Jα33 in humans and TRAV1 (Vα19) Jα33 in mice) paired with variety of TCR β chains (mostly TRBV20 (Vβ2), TRBV6 (Vβ13), in humans and TRBV19 (Vβ6), TRBV13 (Vβ8), in mice) [113, 114]. The expression of CD4/CD8 co-receptors on MAIT cells is different from conventional T cells. In human peripheral blood, most MAIT cells are $CD8^+CD4^-$ single positive (SP) or CD4-CD8- double negative (DN), with lower proportions of $CD4^+CD8^-$ SP or $CD4^+CD8^+$ double positive (DP) cells [115, 116]. In mice, the majority of MAIT cells are DN, and the proportions of $CD4^+$ SP and $CD8^+$ SP subsets vary in different organs [114, 117].

MAIT cells can be found in many sites of the body, though they are named "mucosal-associated" [114, 118, 119]. MAIT cells comprise up to 10% of the peripheral blood T cells in human and are found in high frequency in other organs like the gastrointestinal tract, liver and lungs [119-122]. However, they are present in relatively low frequencies in specific pathogen-free (SPF)-housed laboratory mice, usually only about 0.1% of the peripheral blood T cells [114, 117].

1.3.1 Development of MAIT cells

MAIT cells develop in the thymus and continue to mature after they enter the periphery. They go through selection dependent on MR1-expressing DP cortical thymocytes [123], which is different to conventional T cells selected by thymic epithelial cells. One study divided the development process of MAIT cells into three stages according to the expression of CD24 and CD44 (stage-1 $CD24^+CD44^-$, stage-2 $CD24^-CD44^-$ and stage-3 $CD24^-CD44^+$) [124]. At each stage, MR1 is required for successful development [124]. The promyelocytic leukemia zinc finger (PLZF), which can be found on WT mice MAIT cells [114, 125], is expressed from stage-2. PLZF is crucial for the transition from stage-2 to stage-3 [124].

Most MAIT cells leave the thymus at stage-3, with some exiting by stage-2. After they leave the thymus, they undergo further maturation in the periphery and show the

expression of T-bet or retinoic acid-related orphan receptor γt (RORγt) when they become stage-3 cells, indicating the capacity to secrete cytokines such as IL-17 [124].

1.3.2 MR1 and antigens

The TCRs of MAIT cells are restricted to MR1, which was first described in 1995 [126]. MR1 presents antigens to MAIT cells and is necessary for MAIT cells' development [124]. It is highly conserved in mammals, and this strong evolutionary conservation may suggest an important role in host immune responses [127]. The *Mr1* gene is non-polymorphic and widely expressed in multiple tissue cells, but only low levels of MR1 are detected on the cell surface [128, 129], as the majority (up to 95%) of MR1 remains intracellularly in an incompletely folded form. When there is ligand present, the MR1 folds completely and forms the complex with ligand and β_2-microglobulin (β_2m) [130]. This complex can be transported to cell surface and present the antigen to MAIT cells [130, 131].

The first discovered ligand of MR1 was 6-formylpterin (6-FP), which is a vitamin B9 derivative [132]. 6-FP can form complexes with MR1, but it showed non-stimulatory properties for MAIT cells. Based on the study of MR1-6-FP complex, other stimulatory ligands were identified. The currently known stimulatory ligands include 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe), and 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH) [132, 133], among them, the most potent activator of MAIT cells is 5-OP-RU. These ligands are derived from riboflavin (vitamin B2) synthetic pathway and closely related with 6-FP structurally. This pathway is conserved in bacteria and fungi [134] but not found in virus or mammals.

The discovery of these ligands led to the invention of a key reagent: MR1:5-OP-RU tetramers [114], which can distinguish MAIT cells from other T-cells efficiently and accurately. Before the application of tetramers, the definition of MAIT cells was imprecise in human ($CD3^+V\alpha7.2^+CD161^{hi}$), as the expression of these markers are shared by other T cells (e.g. NK cells) and can change under certain circumstances [116, 135]. In mice, it was even harder because of the unavailability of an anti-Vα19 antibody to date,

which make MR1:5-OP-RU tetramers more important to allow MAIT cell detection in mice in various organs [114, 117].

1.3.3 Activation of MAIT cells

MAIT cells can be activated in a TCR-dependent (antigen specific) or TCR-independent (cytokine driven) manner [136-138]. TCR-dependent activation requires antigens produced from the riboflavin synthetic pathway [139, 140], many riboflavin-producing microbes activate MAIT cells during infection, such as *Salmonella* Typhimurium [117], *Mycobacterium bovis* (*M. bovis*) Bacillus Calmette-Guérin (BCG) vaccine [105], *Legionella longbeachae* (*L. longbeachae*) [141], *Escherichia coli* [139], *Mycobacterium spp.* [142, 143], and *Francisella tularensis* (*F. tularensis*) live vaccine strain (LVS) [144]. This response is abolished when MR1 is blocked with anti-MR1 antibodies [139]. In some riboflavin auxotrophic microbes, such as *Listeria monocytogenes* [140], *Enterococcus faecalis* [139] and *Streptococcus pyogenes* [139], TCR-dependent activation of MAIT cells was not observed, consistent with the riboflavin synthesis as the source of antigens. Besides antigens, previous research showed this TCR-dependent activation also needs the presence of cytokines such as IL-12, IL-18 and IL-23 [138, 145, 146] and other costimulatory signals such as ICOS [146]. In addition to TCR-dependent activation, it is reported that some cytokines can drive MAIT cell activation without TCR-antigen, such as IL-18 combined with IL-12 [137], or IL-18 combined with IL-12, IL-15, and/or the type I interferons IFN-α/β [147, 148]. MAIT cells activated in these two ways possess distinct transcriptional profiles, they all participate in antimicrobial responses but more tissue-repair functions were found related to TCR-dependent activated MAIT cells [136].

Once activated, MAIT cells upregulate the expression of early activation markers such as CD69 and CD107a and produce several cytokines including IFN-γ, IL-17, TNF and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) [118, 149], as well as releasing some cytotoxicity factors like granzyme B and perforin [119, 150]. With different stimuli, MAIT cells can develop into different subsets according to the transcription factor they express: MAIT-1 (RORγt^{low} T-bet^{hi}) and MAIT-17 (RORγt^{hi}), which are closely associated with Th1, Th17 function respectively [119, 151]. With a MAIT-1 phenotype, MAIT cells express the transcription factor T-bet, and produce IFN-

γ and TNF [152]; with MAIT-17 phenotype, MAIT cells express RORγt and produce IL-17 [153].

1.3.4 Function of MAIT cells

Functions of MAIT cells have been widely studied in animal infection models and human diseases. Previous research demonstrated human MAIT cells' response to bacterial infection [139, 152, 154], parasite infection [155], viral infection [148], and many non-infectious diseases, including autoimmune diseases, graft-versus-host disease after transplantation and cancer [156-158]. In several bacterial infection studies, reduced frequency of patients' circulating MAIT cells was observed by comparing with blood samples from healthy donors, such as the study with *Helicobacter pylori* [159], *Shigella dysenteriae* [160], and *Mycobacterium tuberculosis* [139]. However, the exact role of MAIT cells in these diseases is not fully understood.

In animal studies, the antibacterial function of MAIT cells is the most well recognized. The studies of mouse models with some bacterial *in vivo* infections indicating the protective role of MAIT cells by showing the impaired clearing ability of *Mr1*$^{-/-}$ mice, a mouse strain which lacks MAIT cells [141, 144]. Other mouse strains to study MAIT cells include the MAIT TCR-transgenic (Tg) Vα19iTg mice which have significantly higher numbers of MAIT cells, achieved by overexpressing the invariant TCRα genes (Vα19i) and/or TCRβ gene (Vβ6/8), which are used by MAIT cells [161]; and CAST/EiJ mice which have 20 times more frequent MAIT cells than C57BL/6J mice, achieved by the higher usage of Vα19 caused by one CAST genetic trait [125].

Chua, *et al.* demonstrated that MAIT cells have unique innate functions by using the *M. bovis* BCG infection mouse model. They co-cultured *M. bovis* BCG-infected macrophages with naïve T cells from *Mr1*$^{-/-}$, WT, Vα19iTg*Mr1*$^{-/-}$, and Vα19iTg*Mr1*$^{-/-}$ mice, and found activated MAIT cells can produce IFN-γ and IL-17A, and the effector function is dependent on the innate signal IL-12 from infected macrophages. Subsequent *in vivo* experiments showed MAIT cells' non-redundant function which can control bacterial growth at the early stage of infection [152]. Another study suggested MAIT cells' critical and essential role for full resistance to *F. tularensis* LVS. Robust accumulation of MAIT cells was observed after *F. tularensis* LVS intranasal infection with IFN-γ, TNF

and IL-17 production, which were believed to control *F. tularensis* LVS bacterial growth in macrophages. In addition to the secretion of important cytokines, MAIT cells were found to modulate other immune cells, for instance, modulation of monocyte-derived DCs, then recruitment of more activated $CD4^+$ T cells to the lungs during the infection of *F. tularensis* LVS. All of these significantly helped bacterial clearance in WT mice compared to LVS-infected $Mr1^{-/-}$ mice [144].

Unlike studies on bacteria, few studies on the role of MAIT cells have been carried out in fungal infections. Recently, Jahreis, *et al.* have demonstrated that human MAIT cells can be activated *in vitro* by Aspergillus [162], suggesting a role of MAIT cells in fungal infections. In order to test whether Aspergillus can activate MAIT cells *in vivo*, an *A. fumigatus*-infection mouse model needs be established and used to investigate the role of MAIT cells in detail.

1.4 *Aspergillus fumigatus*

Aspergillus species are very common saprophytic fungi, which grow on decaying organic matter [163]. They produce asexual spores (conidia), and some species have a sexual stage [164]. Aspergillus was first isolated in 1729 by the Italian priest and biologist Pier Antonio Micheli. He named the genus according to the shape of their conidia-forming structure, which reminded him of aspergillum, the holy water sprinkler [163]. Aspergillus now is a genus consisting of hundreds of mold species, some of them are opportunistic pathogens. Among them, *Aspergillus fumigatus* (*A. fumigatus*) is the most common species that can cause invasive aspergillosis [165], the most dangerous disease caused by Aspergillus.

1.4.1 Distribution and asexual life cycle

Aspergillus species are highly aerobic and can grow and survive over a wide range of temperatures (from 25°C to above 37°C) and pH. They can be isolated from almost all oxygen-rich environments, such as garden soil, decaying vegetation and even the air [166]. Typically, Aspergillus is in an asexual stage and produces conidia on specialized hyphal structures. These conidia are easily dispersed in the air because they are hydrophobic and small, only 2-3 um in size, which makes them very easy to be inhaled deep in the lung.

On average, one person inhales a few hundred conidia each day [167, 168]. Healthy people are able to completely remove inhaled spores through the airway epithelial cell cilia and alveolar macrophages [169], as well as other defense mechanisms of the immune system [170, 171]. For those (e.g. immune compromised patients) who are unable to remove inhaled conidia, conidia may germinate in the body and cause infections: Aspergillosis [172].

1.4.2 Aspergillosis

Aspergillosis is associated with a range of immune system disorders and is divided into two categories: non-invasive and invasive. People who have pre-existing lung injury and chronic lung disease are at risk for non-invasive aspergillosis. For example, in patients with cavitary lung disease (such as emphysema or previous cavitary tuberculosis) inhaled spores may grow in pre-existing cavities and cause a fungal disease called aspergilloma [173]. In this disease, the fungal hyphae form biofilms [174], and can be treated with surgical resection. In asthma patients, Aspergillus *spp.* can cause allergic disease, resulting in severe allergic bronchopulmonary aspergillosis (ABPA) [175] or severe asthma with fungal sensitization (SAFS) [176].

Immunodeficiency is a major predisposing factor in the development of invasive aspergillosis. Individuals who have advanced acquired immunodeficiency syndrome, chronic granulomatosis or neutropenia, and recipients of hematopoietic stem cell transplants or solid-organ transplants, have much higher risk of invasive aspergillosis [172]. Accurate diagnosis of invasive aspergillosis is still difficult in the clinic. The manifestations are not specific, and both imaging diagnosis and sample cultures lack accuracy and sensitivity. For treatment, voriconazole and amphotericin B deoxycholate (with significantly improved survival of 71% and 58% respectively [177]) are mainly used as initial therapy of invasive aspergillosis. Other treatments, such as drug combination therapies, have also shown some encouraging findings [178].

Elucidating the role of MAIT cells in anti-Aspergillus protection may provide important insights into the prevention and treatment of aspergillosis and other fungal infectious diseases, which are more likely to result in invasive disease in immunocompromised individuals [172].

1.4.3 Host defense against Aspergillus

When conidia are inhaled by humans, respiratory epithelial cells act as an anatomic barrier, and the movement of epithelial cilia can expel most of the inhaled spores. Alveolar macrophages, monocytes, and neutrophils are recruited to sites of infection and clean the remaining spores or hyphae [169, 179]. The host's innate recognition of Aspergillus depends on the host PRRs. The cell walls of Aspergillus contain many PAMPs. These PAMPs can be recognized by the PRRs on host cells. One of the most crucial PAMPs of Aspergillus is β-1,3-glucan [180], which is recognized by the C-type lectin receptor (CLR) dectin-1 [181], the PRR on host cells. Other PRRs, such as TLRs are also involved in fungal antigens recognition [182-184]. This recognition is important for both innate host defense and adaptive defense.

When the body gets infected by Aspergillus, innate immune cells are activated and recruited, and phagocytes begin to engulf and clear spores and hyphae. The NADPH oxidase complex in phagocytes plays an important role in both neutrophil-mediated hyphal damage [185] and monocyte or macrophage-dependent light-chain 3 (LC3) associated phagocytosis (LAP) [186-188]. It also has a balancing role in inhibiting inflammation induced by fungal cell wall components. The activation of LAP also associates with dectin-1-mediated β-1,3-glucan recognition [187, 189]. Aspergillus have evolved several mechanisms to escape the innate immune system. Some conidial surface molecules, such as hydrophobin, can mask PAMPs and block the recognition of PRRs [190, 191]. Others, like melanin, can inhibit the NADPH oxidase complex [192]. Aspergillus also secrete some toxic molecules, for example, fumagillin and gliotoxin, which inhibit neutrophils and macrophage functions respectively [193, 194]. Although they developed these innate immune evasion mechanisms, the innate immune system still plays a major role in the removal of Aspergillus.

After the PAMPs are recognized by PRRs, antigens of Aspergillus can be presented by APCs, mainly DCs, to activate adaptive immune cells. Th1 cells [195], Th2 cells [196], Th17 cells [197], Treg cells [198-200] and cytotoxic T cells [201] have been shown to be involved in the host response to Aspergillus. Th2 cells can produce IL-4, IL-5, and IL-13 and contribute to fungal allergy. IL-17 is the signature cytokine of Th17 cells, it functions

in neutrophil recruitment. However, excessive IL-17 response will impair host defense [202, 203].

1.5 Research Aims

MAIT cells are highly conserved in evolution and are abundant in the human body [119-122]. Many previous studies have shown that they play a non-redundant role in bacterial infections [141, 144, 152, 154]. However, there are relatively few studies on the role of MAIT cells in fungal infections. As MAIT cells can be activated by microbial metabolites derived from the riboflavin biosynthesis pathway, which exists in both bacteria and fungi, it is plausible that MAIT cells can play a role in fungal infections.

One previous paper, published near the beginning of this study, showed that human MAIT cells can be activated by Aspergillus *in vitro* [162]. Aspergillus is a ubiquitous fungus that is not pathogenic to most people. However, the threat of Aspergillus infection persists in some populations, such as patients undergoing chemotherapy and/or radiotherapy, and other immunosuppressed patients [172]. Due to the advancement of medical treatment, more and more people with immunodeficiency can live normal lives through some medical aids. Therefore, more and more people are facing the threat of Aspergillus infection, which makes it important to study the characteristics and responses of MAIT cells in Aspergillus infections. Since the *in vivo* environment is much more complicated than *in vitro*, it was necessary to develop an animal model of Aspergillus infection to study the response of MAIT cells in the *in vivo* environment.

Here, a protective role of MAIT cells during Aspergillus infection was hypothesized. Using MR1:5-OP-RU tetramer to specifically detect MAIT cells, this dissertation aimed to 1) study the response of MAIT cells to Aspergillus both *in vitro* and *in vivo* (Chapter 3 and 2) explore the role of MAIT cells in host defence against Aspergillus infection (Chapter 4). A new murine model was developed to achieve the aims. The findings and data presented may provide a foundation to further study and ultimately bring new ideas to clinical treatment.

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Chapter 2 Material and Methods

2.1 Material: reagents, media and buffers

Table 1. Reagents, media and buffers

Name	Description, ingredients, brand or reference
7-Aminoactinomicin D (7-AAD)	Fluorescent intercalator which can bind to DNA, Sigma-Aldrich (AU), Cat # A9400
Anti-mouse Ly6G	The 1A8 monoclonal antibody reacts with mouse Ly6G, BioXCell (AU), Cat # BP0075-1
Collagenase digestion solution	490 ml RPMI with 2.5 mg DNAse I, 1.5 g Type III collagenase and 10 ml FCS
Cytofix/Cytoperm Plus Kit (with GolgiPlug)	BD Bioscience (AU), Cat # 555028
DNAse I	Roche Diagnostics GmbH
FACS wash buffer	PBS containing 1% FCS
Fetal calf serum (FCS)	HyClone, GE Healthcare Life Sciences
Fixable Viability Dyes (FVD) eFluor 780	Viability dye which can be used to irreversibly label dead cells, Thermo Fisher Scientific, eBioscience, Cat # 65-0865-18
Fixation/Permeabilization Kit	BD Bioscience (AU), Cat # 554714
Foxp3/Transcriptional Factor Staining Buffer Set	Thermo Fisher Scientific, eBioscience, Cat # 00-5523-00
Heparin	DBL Heparin Sodium Injection BP (1,000 IU/mL)
ICS Perm wash buffer	BD Perm/wash Buffer from Fixation/Permeabilization Kit, 1:9 diluted to MQ water
mAb 26.5	MR1-specific monoclonal Ab [128], made in-house
MR1: 5-OP-RU-tetramer	Specific staining of MAIT cells, made in-house
MR1:6-FP-tetramer	Block the non-specific staining of MAIT cells, used prior to MR1: 5-OP-RU-tetramer, made in-house
Percoll	Bio-strategy, Cat # GEHE17-0891-01
Phosphate buffered saline (PBS)	Media preparation unit, Peter Doherty Institute for Infection and Immunity
RF-10 media	500 ml RPMI with 60 ml FCS and 30 ml SC

Roswell Park Memorial Institute (RPMI) 1640 media	Thermo Fisher Scientific, Gibco, Life Technologies, Cat # 11875093
Supplementary components (SC)	Containing β-mercaptoethanol, HEPES, L-glutamine, Non-essential amino acid solution, Penicillin-streptomycin solution
TF Perm wash buffer	Permeabilization Buffer from Foxp3/Transcription Factor Fixation/Permeabilization Kit, 1:9 diluted to MQ water
TransIT-EE hydrodynamic delivery solution	MIR 5340, Mirus Bio LLC
Tris-buffered ammonium chloride (TAC) red blood cell lysis solution	PBS containing 46 mM Tris-CI (pH 8.1), 0.1% sodium azide and 1 mM $CaCl_2$
Tween 20	Sigma-Aldrich, Cat # P1379
Type III collagenase	Worthington Biochemical Corporation, Cat # LS004183

2.2 Methods

2.2.1 Cell lines and MR1-dependent MAIT cell activation *in vitro* assay

Jurkat.MAIT cell is a derivatives of Jurkat cell, which is an human immortalized T leukemia cell line [204]. The Jurkat.MAIT cell line is transduced with MAIT cell receptors which express Vα7.2-Jα33 (TRAV1-2-TRAJ33) invariant α-chain with either a Vβ13.3 (TRBV6-1), Vβ13.5 (TRBV6-4), or Vβ2 β-chain (TRBV20) [113]. In this study, Jurkat.MAIT TRBV6-1, Jurkat.MAIT TRBV6-4, and Jurkat.MAIT TRBV20 were used.

C1R.hMR1 cell is a mutant derivative of HMy2.C1R cell [132], which is a human B lymphoblast cell line. C1R.hMR1 cell line is generated by transducing the human *Mr1* gene in to HMy2.C1R cells, making them express high levels of MR1 at the cell surface [132]. C1R.hMR1 cells serve as antigen presenting cells to examine the MAIT cell response.

To assay MR1-dependent MAIT cell activation *in vitro*, Jurkat.MAIT cells (10^5 /well) and C1R.hMR1 cells (10^5 /well) were suspended in RF-10 and transferred into 96 well U-bottom plates. MR1 specific monoclonal antibody (mAb) 26.5 (10 μg/ml) was added

to confirm whether activation is nonspecific or MR1-dependent. Stimuli*[1] (synthetic ligands, filtered fungal culture supernatant or controls) were added and mixed sufficiently by pipetting up and down 4 times.

After co-incubation for 16 hours (37°C, 5% CO_2), cells were then centrifuged (4°C ,400 revolutions per minute (RPM) (37 x *g*)) for 4 minutes, and washed with FACS wash buffer (150 µl/well) twice. Cells were stained with anti-human CD3 and anti-human CD69 for 30 minutes, and then washed with FACS wash buffer before being resuspended in FACS Fix (150 µl/well), and analysed by flow cytometer. The activation of Jurkat.MAIT cells was detected by upregulation of CD69, measured by the Mean Fluorescence Intensity (MFI) calculated using Flowjo software (version 10). In some experiments, anti-MR1 mAb 26.5 (final concentration 20 µg/well) was added 2 hours before stimuli to block MR1 [205].

Table 2. Staining panel of Jurkat.MAIT cells

Antibody	Fluorochrome	Concentration (mg/ml)	Dilution	Clone	Manufacturer
CD3	PE-Cy7	0.1	1:300	UCHT1	eBioscience, Cat # 25-0038-42
CD69	APC	-	1:50	FN50	BD Bioscience, Cat # 555533

2.2.2 Fungal strain and growth conditions

The strains used in this study were *Aspergillus fumigatus* CEA10Δku80 and Af293, which were kindly provided by Professor Alex Andrianopoulos, Department of Genetics, The University of Melbourne.

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*[1]Stimuli in this study include synthetic ligands and filtered fungal culture supernatant. Synthetic ligands: 0.1 nM MAIT cell ligand 5-OP-RU (a potent MAIT cell activator) was included as positive control; 1 µM acetyl-6-formylpterin (Ac-6-FP) (strong MR1 binder but does not activate MAIT cells) was included as a negative control for activation. Filtered fungal culture supernatant was made by adding 10^8 conidia of *A. fumigatus* into 10 ml RF-10 and incubated with shaking for 6 hours, 10 hours and overnight, then strained through a 0.2 µm filter to remove conidia and debris. Other negative controls included: Nil (Jurkat.MAIT cells and C1R.hMR1 cells, with RF-10; because in this experiment the culture media for cell lines and *A. fumigatus* was both RF-10).

CEA10Δku80 is a derivate of a clinical isolate strain CEA10 [206]. It lacks the Ku80 protein, which is required for non-homologous end joining (NHEJ) pathway of DNA repair, thus the Δku80 strain allows homologous recombination to occur at a higher frequency and has been largely used due to the simplicity and convenience to make specific mutant strains. The Δku80 strain is not affected in growth and development, and has similar virulence to its wild type (WT) parental strain CEA10. CEA10Δku80 is a "hyper-inflammatory" strain, it has higher fungal growth and clearance rate in early infection than "inflammatory" strain such as Af293 [207].

Af293 is a clinical strain isolated from a patient with human lung invasive aspergillosis. It is a "inflammatory" stain with lower fungal growth and clearance rate in early infection [207].

Fungus stocks were stored at -80 °C in 0.02% Tween in PBS with 50% glycerol. Growth of *A. fumigatus* was achieved by streaking glycerol stock to Potato Dextrose Agar (PDA) plates with streptomycin (50 μg/ml). Streaking was accomplished by dragging 8 μl stock with inoculation loop in a shape of "✳". The inoculation loop was sterilized by passing through a flame beforehand and between streaks. Plates were then incubated at 37 °C for 7 days to allow maximum conidia yield (around 10^7 conidia per plate). Conidia were collected by adding 2 ml of 0.02% Tween in PBS to each plate and by scraping with plastic spreaders. Conidia were filtered by 30 μm strainer and stored in at 4 °C for three days to remove viable hyphae. *A. fumigatus* conidia remain viable for about a month when stored at 4 °C. Estimation of the stock conidia concentration was performed by plating serial dilution and colony counting. The required volume of conidia (e.g. $3x10^7$ CFU) was centrifuged at 3000 RPM (2095 x *g*) for 10 minutes at 4 °C then resuspended to a volume of 0.02% Tween in PBS for a desired infection dose.

2.2.3 Mice and inoculations

C57BL/6 mice are a common inbred strain laboratory mouse and express WT levels of MR1.

C57BL/6.*Mr1*$^{-/-}$ mice [120], abbreviated as *Mr1*$^{-/-}$ mice in this thesis, were generated by breeding Vα19iCα$^{-/-}$ *Mr1*$^{-/-}$ mice [161] with WT C57BL/6 mice followed by inter-

crossing of F1 mice. *Mr1*$^{-/-}$ mice lack *Mr1*. Since MR1 is required for successful development of MAIT cells, *Mr1*$^{-/-}$ mice are deficient in MAIT cells but other functions are intact, which making them a good control to analyze MAIT cell functions [120].

Il-23p19$^{-/-}$ mice [208] are mice deleted *Il-23p19* gene. The deletions result in a deficient of cytokine IL-23.

Mice were bred and housed in the Biological Research Facility of Peter Doherty Institute for Infection and Immunity (Melbourne, Victoria, Australia).

All 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: 1814616, and 1814662).

C57BL/6 mice, *Mr1*$^{-/-}$ mice or *Il-23p19*$^{-/-}$ mice aged 6-14 weeks were infected by different routes (except for experiments with hydrodynamic injection, 6-7 weeks old male mice were used in most experiments), including intravenous (i.v.), and intratracheal (i.t.). Intravenous infections were achieved by injecting 200 µl of prepared fungal inoculum into the tail vein with an insulin syringe after warming the mice for 10 minutes (max to five mice were put into a standard M1 mouse cage with lid, and placed on a heat box with two standard 60-watt globes). Intratracheal infections were achieved by dispensing 50 µl of prepared fungal inoculum on anesthetized mice with a pipette and gel loading tip into the tracheal. Mice were anaesthetised by isoflurane inhalation administered using an anaesthetic machine generating an isoflurane/oxygen mix (2 L/min Oxygen flow, isoflurane was added at maximum level). When the mouse was unconscious, transferred it to a Class II biosafety cabinet and placed on a platform with its front teeth hanged with copper wire. A laryngoscope was used to locate the tracheal. Mice were monitored accordingly after infection.

Intranasal (i.n.) inoculation was used to deliver zymosan and/or 5-OP-RU to mice in this dissertation. After anaesthetizing by isoflurane inhalation, mice were intranasally

administered 50 μl inocula containing antigens alone (5-OP-RU, 76 pmol per time) or in combination with adjuvants (zymosan, 50 μg). This administration was achieved by instilling the inocula drop by drop to the nostril with a 200 μl pipette.

Intraperitoneal (i.p.) administration was achieved by injecting 200 μl of cyclophosphamide (CTX) (150 mg/kg) inoculum with an insulin syringe into the lower right quadrant of the abdomen of mice.

Subcutaneous (sc) administration was achieved by injecting 200 μl of cortisone acetate (CA) (200 mg/kg) inoculum with an insulin syringe. After anaesthetizing mouse by isoflurane inhalation, inocula was injected into the subcutaneous of its neck fluently. Aspirated the syringe before injection to make sure needle did not penetrate the blood vessel.

Hydrodynamic injection was used to deliver IL-23–Ig plasmid (pEF-BOS–IL-23–IgG3) to mice, IL-23–Ig plasmid constructs were provided by Professor Burkhard Becher, Switzerland [146, 209]. It was used previously to study IL-23 functions in MAIT cell activation [146]. Hydrodynamic injection was performed by injected 1.7 ml of TransIT-EE Hydrodynamic Delivery Solution which contain 10 mg plasmid vector encoding IL-23–Ig to prewarmed mice through tail vein. Female mice aged 6-7 weeks were preferred to conduct this operation.

2.2.4 Mouse euthanasia and organ harvest

After infection with *A. fumigatus*, mice were weighed daily and rated for visual signs of morbidity. In this study, humane endpoints were defined by weight loss (below 80% of initial weight) and behavioural changes (such as walking in a circle, inactivity, laboured breathing and huddling). When the above signs were identified, humane killing by CO_2 euthanasia was carried out to alleviate pain and/or distress.

At the endpoints of experiments, mice were euthanized by administration of CO_2 and organs: lung (following perfusion with RPMI from ventriculus dexter), liver (following perfusion with RPMI from the portal vein), blood (from heart and mixed with 10 μl of

Heparin) and spleen were removed by dissection. Organs were collected into plates with RPMI in the wells.

2.2.5 Assessment of fungal loads by CFU analysis

The lungs of infected mice were homogenized by homogenizer (polytron® PT 2500 E, Thermo Fisher Scientific) for 20 seconds, 21,000 RPM in 0.02% Tween in PBS (6 ml for whole lungs; 3 ml for half lungs). The lysate was diluted with 0.02% Tween in PBS, and then 100 µl of the diluted lysate was spread on a PDA plate with a sterile plastic spreader. Plates were wrapped cling wrap and incubated at 37 °C until colonies were visible, usually 2 days. Colony-forming unit (CFU) data was obtained by counting colonies and used to calculate the fungal burden in infected organs. Plates with a colony number between 20 and 400 were selected during the analysis to ensure accuracy.

2.2.6 Preparation of single cell suspension from organs

Single-cell suspensions were prepared from each organ according to the respective protocols:

Lungs were cut with a surgical scalpel blade to fine pieces and digested with collagenase III and DNase (3 mg/ml collagenase III, 5 mg/ml DNase, and 1% FCS in RPMI) for 90 minutes at 37 °C with gentle shaking (120-180 RPM). The digest was then passed through 70 µm strainers, followed by red blood cell lysis with 3 ml hypotonic buffer TAC (Tris-based amino chloride) for 5 minutes at room temperature. Then 3 ml FACS wash was added to stop TAC reaction. Centrifuged (1400 RPM (456 x *g*), 5 minutes, 4 °C) and aspirated the supernatant, then resuspended with 5 ml FACS wash.

Livers were pushed through 70 µm strainers, followed by red blood cell lysis as described above. White blood cells (including MAIT cells) were separated and collected via density gradient centrifugation (2000 RPM (931 x *g*), 18 minutes, room temperature) with 36% and 72% Percoll diluted in PBS. Then centrifuged (1400 RPM (456 x *g*), 5 minutes, 4 °C) and resuspended with 1 ml FACS wash.

Spleens were pushed through 70 µm strainers and followed by red blood cell lysis as described above. Centrifuged (1400 RPM (456 x *g*), 5 minutes, 4 °C) and resuspended with 5 ml FACS wash.

Blood samples were prepared by twice performing red blood cell lysis with TAC. And resuspended with 1 ml FACS wash.

2.2.7 Surface and intracellular staining

2.2.7.1 Surface staining

Before staining, cells (approximately $1.5x10^6$) were treated with 20 µl of blocking solution for 15 minutes at room temperature to block non-specific antibody/tetramer binding. The blocking solution contained the Fc receptor antibody (clone 2.4G2, neat hybridoma supernatant) and unlabelled MR1:6-FP tetramer*[2] (when staining MAIT cells). Then cells were then stained with 40 µl antibody cocktail for 30 minutes at room temperature. Samples were kept in the dark during the staining process and vortexed every 15 minutes.

After staining, cells were washed (added wash buffer, vortexed, then centrifuged at 1400 RPM (456 x *g*) for 5 minutes, and aspirated the supernatant) twice with 1 ml FACS wash buffer and resuspended in FACS Fix. Added $3x10^4$ blank calibration beads before flow cytometry was performed on Fortessa Flow cytometer. Acquired cellular data were analysed in Flowjo (version 10), Excel, and Prism (version 8). Statistical analysis was performed as appropriate and statistical significance was displayed in figures as defined in the legends.

Table 3. Staining panel of murine MAIT cells

Antibody	Fluorochrome	Concentration (mg/ml)	Dilution	Clone	Manufacturer
-	7-AAD	0.05	1:500	-	BioLegend, Cat # 420404
CD45.2	FITC	0.5	1:200	104	BD Bioscience, Cat # 553772
CD19	PerCP-Cy5.5	0.2	1:200	1D3	BD Bioscience, Cat # 551001

TCRβ	APC	0.2	1:200	H57-597	BD Bioscience, Cat # 553174
MR1: 5-OP-RU tetramer*[3]	BV421	0.185	1:200	N/A	Made in house
CD4	APC-Cy7	0.2	1:200	GK1.5	BD Bioscience, Cat # 552051
CD8α	PE	0.2	1:1600	53-6.7	eBioscience, Cat # 12-0081-83

Table 4. Staining panel of murine innate immune cells

Antibody	**Fluorochrome**	**Concentration (mg/ml)**	**Dilution**	**Clone**	**Manufacture**
	7-AAD	0.05	1:500	-	BioLegend, Cat # 420404
CD45	BV605	0.2	1:200	30-F11	BD Bioscience, Cat # 563053
Ly6G	PE-Cy7	0.2	1:200	1A8	BD Bioscience, Cat # 560601
CD11b	FITC	0.5	1:200	M1/70	BD Bioscience, Cat # 553310
CD103	BUV395	0.2	1:200	M290	BD Bioscience, Cat # 740238
F4/80	APC	0.2	1:200	BM8	BioLegend, Cat # 123116
MHCII	BV421	0.05	1:200	M5/114.15.2	BioLegend, Cat # 107631
CD11c	PE	0.2	1:200	N418	BioLegend, Cat # 117308

2.2.7.2 Intracellular cytokine staining (ICS)

For the lung, GolgiPlug (1:1000) was added during digestion. Before staining, cells (approximately $1.5x10^6$) were stimulated with 20 ng/ml PMA (Phorbol 12-myristate 13-acetate) and 1 µl/ml ionomycin *ex vivo* for 3 hours (37 °C, 5% CO_2). Then washed with 4 ml PBS and stained with 400 µl FVD (Fixable Viability Dyes) (1:1000 diluted in PBS) on ice for 40 minutes in dark. Cells were then washed twice with 1 ml FACS wash and added 20 µl of blocking solution for 15 minutes at room temperature to block non-specific antibody binding. Then cells were stained for surface markers as previously described.

*[2] MR1:6-FP tetramer was prepared by tetramerizing MR1:6-FP monomers with Streptavidin (SA) (molar ratio at 4:1) as described previously [113, 133].

*[3] 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 [113] and specifically stains MAIT cells.

After surface staining, cells were washed with 1 ml FACS wash buffer, and added 250 µl BD cytofix/cytoperm Fixation and Permeabilization Solution for 20 minutes. Washed with ICS Perm wash buffer (BD Perm/wash 1:9 diluted to MQ water). Then stained with 100 µl antibody cocktail overnight at 4 °C in dark (antibodies were diluted with ICS Perm wash). After staining, cells were washed twice with 1 ml ICS Perm wash buffer and resuspended in FACS Fix. Cytokine staining in this study was based on instruction manuals provided in the Fixation/Permeabilization Kit by BD Bioscience.

Table 5. Staining panel of cytokines of murine MAIT cells

Antibody	Fluorochrome	Concentration (mg/ml)	Dilution	Clone	Manufacture
FVD	eFluor 780	-	1:1000	-	eBioscience, Cat # 65-0865-14
CD45.2	FITC	0.5	1:200	104	BD Bioscience, Cat # 553772
CD19	PerCP-Cy5.5	0.2	1:200	1D3	BD Bioscience, Cat # 551001
TCRβ	APC	0.2	1:200	H57-597	BD Bioscience, Cat # 553174
MR1: 5-OP-RU tetramer	BV421	0.185	1:200	N/A	Made in house
IL-17A	PE	0.2	1:200	TC11-18H10	BD Bioscience, Cat # 559502
IFNγ	PE-Cy7	0.2	1:400	XMG1.2	BD Bioscience, Cat # 557649
GM-CSF	PE	0.1	1:200	MP1-22E9	BD Bioscience, Cat # 554406
TNF	PE	0.1	1:200	MP6-XT22	BD Bioscience, Cat # 554419

2.2.7.3 Transcription factor (TF) staining

Cells (approximately $1.5x10^6$) were washed with 4 ml PBS and stained with 400 µl FVD (1:1000 diluted in PBS) on ice for 40 minutes. After staining, cells were washed twice with 1 ml FACS wash. Then 20 µl of blocking solution was added for 15 minutes at room temperature to block non-specific antibody binding. Then cells were stained for surface markers as previously described.

After surface staining, cells were washed with 1 ml FACS wash buffer. Fixation/Permeabilization Concentrate was diluted in Fixation/Permeabilization Perm Diluent (1:3), and added to sample (400 µl/well) for 1 hours at 4 °C in dark. Washed with

TF Perm wash buffer (Permeabilization Buffer 1:9 diluted to MQ water). Then stained with 100 µl antibody cocktail for 1 hour at 4 °C in dark (antibodies were diluted with TF Perm wash). After staining, cells were washed twice with 1 ml ICS Perm wash buffer and resuspended in FACS Fix.

Transcription factor staining in this study was performed by following manufaturer's instructions provided in the Foxp3/Transcription Factor Fixation/Permeabilization Kit from eBioscience.

Table 6. Staining panel of transcription factors of murine MAIT cells

Antibody	Fluorochrome	Concentration (mg/ml)	Dilution	Clone	Manufacture
FVD	eFluor 780		1:1000		eBioscience, Cat # 65-0865-14
CD45.2	FITC	0.5	1:200	104	BD Bioscience, Cat # 553772
CD19	PerCP-Cy5.5	0.2	1:200	1D3	BD Bioscience, Cat # 551001
TCRβ	PE	0.2	1:200	H57-597	BD Bioscience, Cat # 553172
MR1: 5-OP-RU tetramer	BV421	0.185	1:200	N/A	Made in house
RORγt	APC	0.2	1:200	B2D	eBioscience, Cat # 17-6981-82
T-bet	PE-Cy7	0.2	1:200	eBio4B10 (4B10)	eBioscience, Cat # 25-5825-82

2.2.8 Gating strategy for MAIT cells

Flow cytometric analysis of stained samples was performed using a BD LSRFortessa. FACS files were analysed using Flowjo software (version 10, Flowjo LLC). The gating strategy for MAIT cells is shown in **Figure 1**.

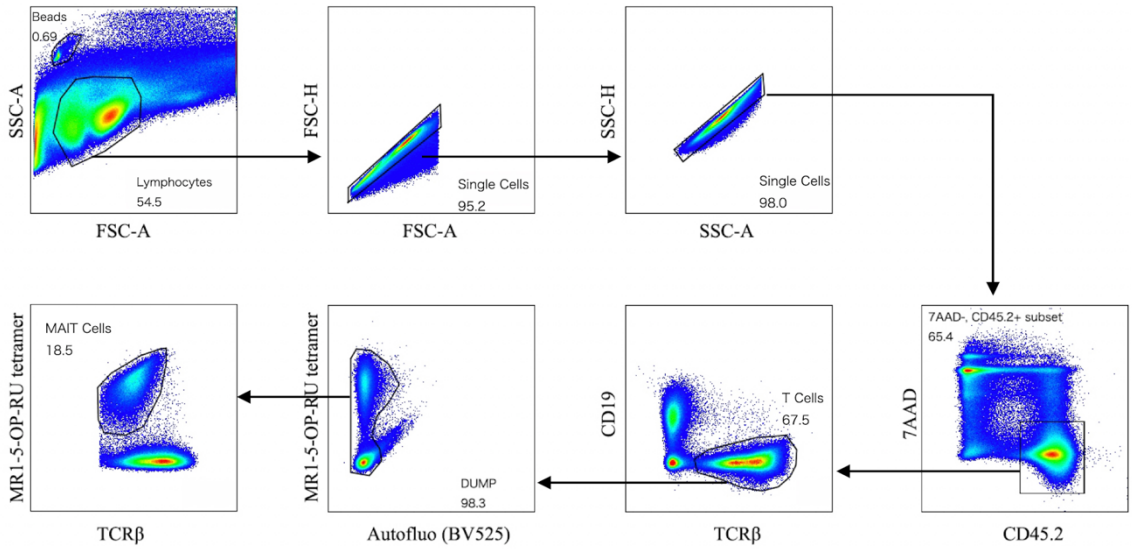


Figure 1. Gating strategy for MAIT cells

Mouse MAIT cells were defined as 7-AAD$^-$, CD45.2$^+$, TCRβ$^+$, MR1: 5-OP-RU tetramer$^+$ population. Beads and lymphocytes were gated by forward versus side scatter, and single cells discriminated from scatter area versus height. Live lymphocytes were further gated as 7-AAD$^-$ CD45$^+$ population, T lymphocytes were then elected as TCRβ$^+$ population. Autofluorescent cells were excluded by displaying cells with Violet 525 vs Violet 50, which flank BV421. MAIT cells were specifically stained with MR1: 5-OP-RU tetramer.

2.2.9 Statistical analysis

All statistical tests were performed using Prism GraphPad software (Version 8). Comparisons between groups were performed using unpaired t test, unless otherwise stated.

Table 7. Statistical tests used for Figures

	Statistical test used
Figure 2	Unpaired t test
Figure 3	Unpaired t test
Figure 7	Two-way ANOVA
Figure 10B	Ordinary one-way ANOVA
Figure 11B	Unpaired t test
Figure 12	Two-way ANOVA
Figure 14C	Ordinary one-way ANOVA
Figure 18	Two-way ANOVA

Chapter 3 The Activation of MAIT cells by *Aspergillus fumigatus in vitro* and *in vivo*

3.1 Introduction

Previous research has demonstrated that MAIT cells respond to a range of bacterial infections [117, 139, 141, 144, 152, 154]. And the protective roles were found in infections of *L. longbeachae* [141], *M. bovis* BCG [152], *F. tularensis* live vaccine strain (LVS) [144], and *Klebsiella pneumoniae* [154]. However, for fungal infections, there are few studies that assess the role of MAIT cells [139, 210]. Since the riboflavin synthetic pathway that generates MAIT cell antigens is present in both bacteria and fungi [134], and fungal infections are also very common [211], it is necessary to study the role of MAIT cells in fungal infection.

A. fumigatus is an opportunistic pathogen which has an intact riboflavin synthetic pathway [212]. The conidia of *A. fumigatus* are widely dispersed in air [167]. It enters the body by the respiratory tract and typically only causes disease in people with immunodeficiency and specific lung conditions [213]. In 2018, there was one study by Jahreis, *et al.* which showed that human MAIT cells can be activated by *A. fumigatus, in vitro,* by coincubation with conidia [162]. Here, a well-established *in vitro* system was employed [132] to detect if *A. fumigatus* produce MAIT cell ligands. Furthermore, to investigate the function of MAIT cells in *A. fumigatus* infection *in vivo*, a mouse model of pulmonary infection was established using two strains of *A. fumigatus* (Af293 and CEA10Δku80**)**. *A. fumigatus* Af293 is a widely used strain in animal models [214-218]. It is described as "inflammatory" strain in Rizzetto's study [207], which means lower fungal growth and fungal clearance rate in early infection compared with "hyper-inflammatory". *A. fumigatus* CEA10Δku80 has similar virulence to the "hyper-inflammatory" stain CEA10 [206]. These two selected strains can represent the two major categories of heterogeneous *A. fumigatus* strains [207].

MAIT cells immune response (activation, kinetics and accumulation) during the infection of *A. fumigatus* (Af293 and CEA10Δku80**)** was investigated using the pulmonary infection murine model. MAIT cells were identified by flow cytometry using MR1:5-OP-RU-tetramers, in combination with T cell markers.

3.2 Results

3.2.1 *A. fumigatus* metabolites activate human MAIT cells *in vitro*

In order to confirm *in vitro* activation of MAIT cells by *A. fumigatus* as previously shown in a different system by Jahreis, *et al.* [162], an activation assay was performed by coculture of reporter cells and APCs with *A. fumigatus* culture supernatant (S/N). The reporter cell lines used were Jurkat.MAIT cells, which are Jurkat cells (an immortalized line of human T leukemia cells) transduced with MAIT cell receptors which express Vα7.2-Jα33 (TRAV1-2-TRAJ33) invariant α-chain with either a Vβ13.3 (TRBV6-1), Vβ13.5 (TRBV6-4), or Vβ2 β-chain (TRBV20). The APC line, C1R.hMR1 cells, were generated by transducing the human *Mr1* gene into the human lymphoblastoid B cell line. C1R.hMR1 cells express high levels of MR1 at the cell surface, making them suitable APCs to examine the MAIT cell response [219].

To make the *A. fumigatus* S/N, Potato Dextrose Broth (PDB) were chosen to grow the conidia. However, the culture supernatant from PDB could not activate Jurkat.MAIT cells with the *in vitro* assay (data not shown). Then RF-10, a commonly used cell culture medium, was introduced to make *A. fumigatus* supernatant.

The activation of Jurkat.MAIT cells was assessed by measuring the cell surface expression level of CD69, which reflect by the mean channel fluorescence intensity (MFI). Similar to synthetic 5-OP-RU, the potent MAIT cell activator, addition of *A. fumigatus* supernatant (both Af293 and CEA10Δku80) to the co-culture assay resulted in significant upregulation of CD69 expression on all three Jurkat.MAIT cell lines tested compared to negative Nil (no sample) controls. When MR1 blocking antibody was added, the activation driven by Aspergillus metabolites was significantly impaired, suggesting the activation is MR1-dependent (**Figure 2**).

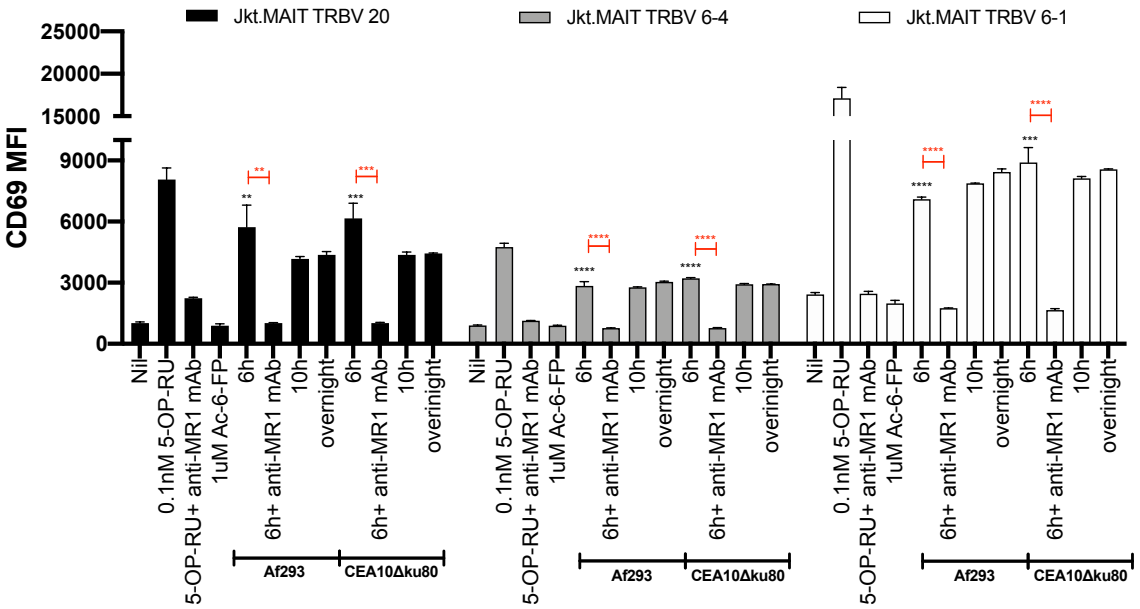


Figure 2. The metabolites of *A. fumigatus* activated Jurkat.MAIT cells *in vitro*

Jurkat.MAIT cells (3 lines, 10^5 per well) and C1R.hMR1 cells (10^5 per well) were co-incubated with 20 µl *A. fumigatus* Af293 or *A. fumigatus* CEA10Δku80 culture supernatants (harvested at 6 hours or 10 hours or overnight as indicated), RF-10 media, Ac-6-FP or 5-OP-RU with/without anti-MR1 mAb (mAb 26.5, 10 µg/ml final, added 2 hours before addition of supernatant or control ligands). After stimulating Jurkat.MAIT cells in an overnight co-culture assay, cells were stained with anti-CD3, and anti-CD69. Activation was assessed by the MFI of CD69 on gated Jurkat.MAIT cells.

Data from two independent experiments within two weeks with similar results, n=4; mean ± SEM. **P < 0.01, ***P < 0.001, **** P < 0.0001 calculated by unpaired t test.

The above results confirmed that *A. fumigatus* can produce MAIT cell-activating ligands presented by MR1. Based on previous research, it would be reasonable to assume these ligands originate from the riboflavin synthesis pathway and hypothesize that the above tested strains of *A. fumigatus* are able to activate MAIT cells *in vivo*.

3.2.2 *A. fumigatus* activates MAIT cells in C57BL/6 mice *in vivo*

To study *A. fumigatus* infection *in vivo*, a consistent dosage of *A. fumigatus* conidia was needed to infect mice. As the fungal conidia are hydrophobic and often aggregate when mixed with aqueous solutions such as PBS, a detergent was used to facilitate dissolution. After serial titration, 0.02% Tween 20 in PBS was used to facilitate the dissolution and a homogenous inoculum was produced, allowing consistent doses of conidia to be delivered to each mouse. Tween is a non-ionic surfactant which is easily soluble in water. It was already used in many *A. fumigatus* studies to suspend the conidia during infections or colony counting [220].

To test the potential effects of 0.02% Tween in PBS on the murine MAIT cell response, C57BL/6 mice were given 0.02% Tween in PBS intratracheally and MAIT cells were examined on day 6 (**Figure 3**).

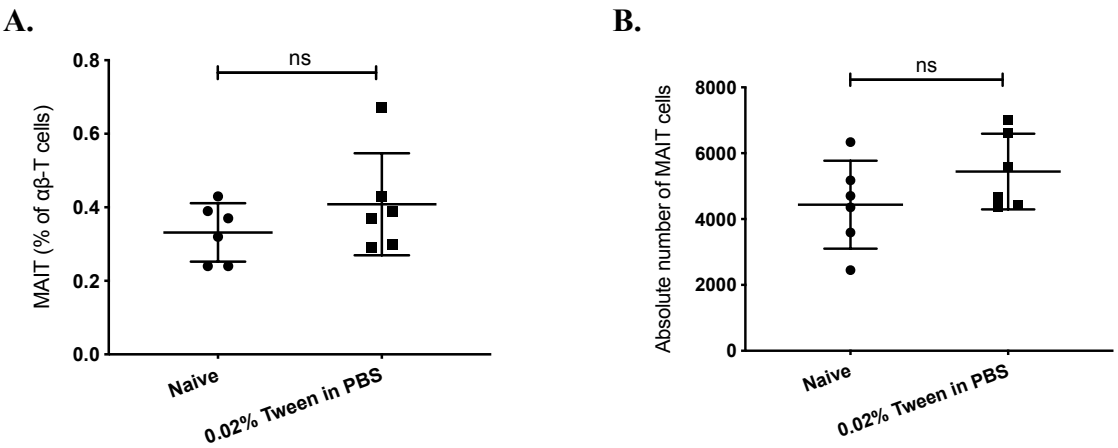


Figure 3. No detrimental effect of 0.02% Tween on pulmonary MAIT cells was detected

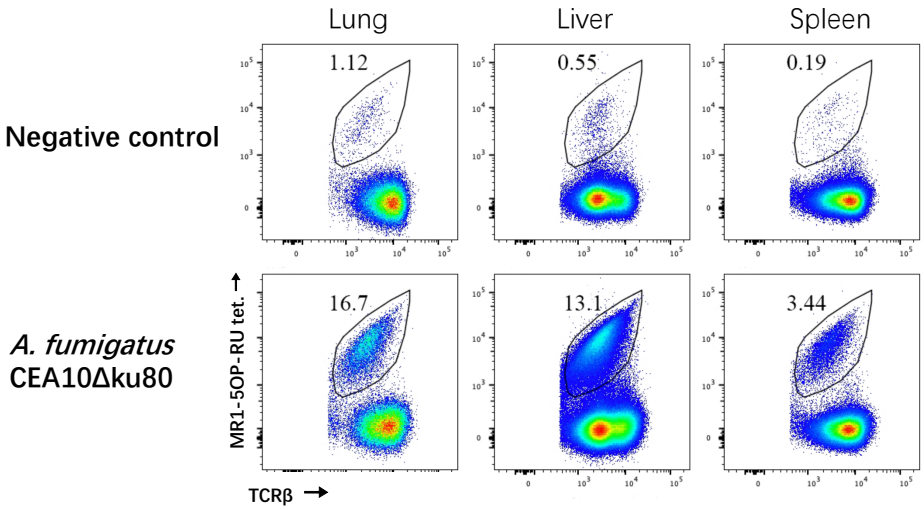
Percentage (A) and absolute numbers (B) of MAIT cells (TCRβ$^+$MR1:5-OP-RU tetramer$^+$) isolated from the lungs of naïve mice or mice treated with 0.02% Tween in PBS (50 µl, intratracheally). Data from two independent experiments, n=6; mean ± SEM. ns $P > 0.05$, calculated by unpaired t test.

By comparing the absolute number and percentage of MAIT cells in naïve mice and 0.02% Tween in PBS-treated mice, we found that Tween did not increase or decrease MAIT cell numbers, suggesting this low concentration (0.02%) has no obvious detrimental effect on MAIT cell accumulation. Therefore, 0.02% Tween in PBS was used throughout all following experiments, including infections, plating, and storage at 4 °C.

The *in vivo* environment is much more complicated than *in vitro*. Ligands such as 5-OP-RU alone are not sufficient to activate MAIT cells *in vivo* [117] and this may also apply to Aspergillus. In order to provide a basis for the murine model establishment, a preliminary assessment of the MAIT cell response of *A. fumigatus in vivo* was conducted. C57BL/6 mice were given *A. fumigatus* CEA10Δku80 conidia intravenously (i.v.) or intratracheally (i.t.). In intravenous infected mice, MAIT cells were tested in the lung, liver, and spleen. All three organs showed an increased MAIT cells percentage of T cells, which was especially apparent in the lung and liver (**Figure 4A**).

Since *A. fumigatus* is an inhaled opportunistic pathogen, intratracheal instillation, rather than intranasal delivery, was used to ensure the conidia could reach deep into the lungs. C57BL/6 mice were infected with a titrating dose (10^5, 10^6 and 10^7 CFU) of *A. fumigatus* CEA10Δku80 conidia. A significant accumulation of pulmonary MAIT cells was observed in 10^7 CFU infected mice, which comprised an average of 10% of all pulmonary αβ T cells on 6 DPI (**Figure 4B**).

A.



B.

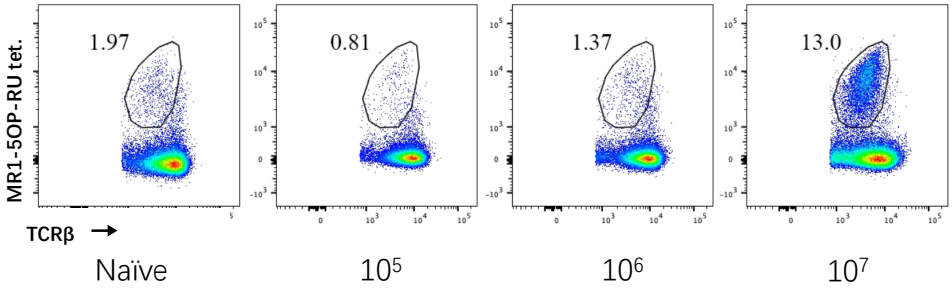


Figure 4. MAIT cells are activated by *A. fumigatus* CEA10Δku80 in C57BL/6 mice *in vivo*

(A) Representative flow cytometry plots showing MAIT cell percentage of total TCRβ$^+$ T cells in the lung, liver and spleen of C57BL/6 mice either uninfected (injected with 0.02% Tween in PBS as negative control) or intravenously infected with 10^6 CFU *A. fumigatus* CEA10Δku80 conidia, MAIT cells were analysed on 6 DPI.

(B) Pulmonary MAIT cell percentage of total TCRβ$^+$ T cells of C57BL/6 mice intratracheally infected with indicated doses of *A. fumigatus* CEA10Δku80 on 6 DPI.

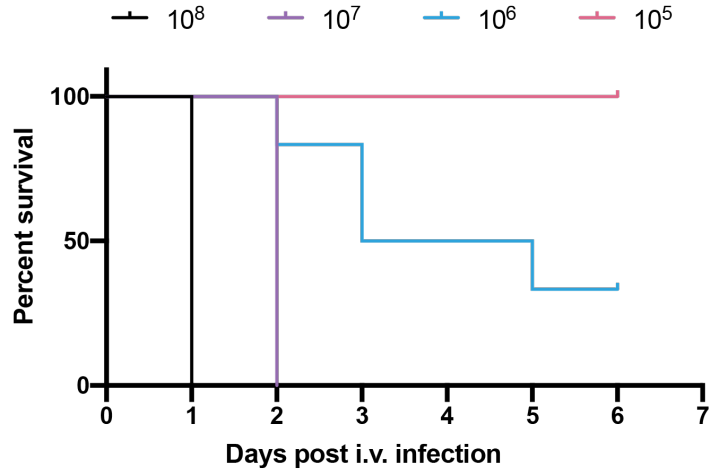
These data demonstrate that *A. fumigatus* can stimulate a MAIT cell response *in vivo* in mice via intravenous and intratracheal infection.

These two routes of administration represent two models of *A. fumigatus* infection. The intravenous injection is a systemic infection model which mimics the infection of patients with Aspergillus in the circulatory system. It is a very serious disease with very little chance of recovery. The second model used intratracheal infection to mimic a localized infection in the lungs. In order to find the best model for this study, serial titrations of *A. fumigatus* conidia (10^5, 10^6, 10^7, 10^8 CFU for intravenous infection and $1.25x10^7$, $2.5x10^7$, $5x10^7$, 10^8 CFU for intratracheal infection) were performed, and survival rate of these two models were analysed (**Figure 5**).

Intravenous infection (**Figure 5A**) showed a high mortality rate (100% in 10^7 and 10^8 CFU infected mice and more than 50% mice in 10^6 infected mice reached humane endpoints). At the time the humane endpoint was reached, we found that most mice showed very severe inner ear infection symptoms, such as walking in a circle and loss of balance. The high mortality rate and the high incidence of inner ear infection symptoms made it impossible to continue the intravenous infection model at a sufficiently high dose to induce MAIT cell accumulation, whilst ensuring that the mice did not suffer these symptoms. Considering the aim of the project was to evaluate the response and the role of MAIT cells in Aspergillus infection to potentially identify targets for the prevention and treatment of aspergillosis, the rarity of systemic infections in the clinic also made the intravenous infection model difficult to provide insights for the widest range of conditions. Thus the intravenous mode of inoculation was ceased and the intratracheal mode was used for all further described experiments.

The intratracheal infected mice showed fewer inner ear infection symptoms and had a higher survival rate with high dosages including $5x10^7$ CFU (**Figure 5B**). This made intratracheal infection the most viable administration method and helped to determine the optimal doses to inform future experiments.

A.



B.

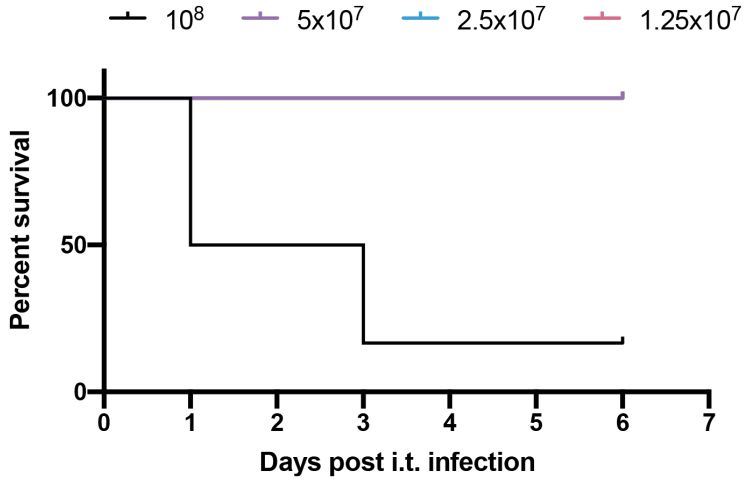


Figure 5. Survival of mice following i.v. or i.t. infection with *A. fumigatus* CEA10Δku80

C57BL/6 mice were infected with *A. fumigatus* CEA10Δku80 conidia intravenously (A) or intratracheally (B) with indicated doses, and the survival (defined as time to reach humane end points) of mice was monitored after infection. The Y-axis shows the survival percentage of mice after infection as a proportion of the original number. The X-axis shows days post infection. (A) For group 10^5 and 10^8, n=3; for group 10^6 and 10^7, n=6. (B) For group $2.5x10^7$, $5x10^7$ and 10^8, n=6; For group $1.25x10^7$, n=3.

3.2.3 Robust accumulation of MAIT cells in *A. fumigatus* intratracheally infected mice

To titrate the inoculation dose, C57BL/6 mice were given $1.25x10^7$, $2.5x10^7$, $5x10^7$ or 10^8 CFU of *A. fumigatus* CEA10Δku80 conidia intratracheally and MAIT cells and non-MAIT conventional αβ T cells analysed in the lungs by flow cytometry at various time points post infection. Accumulation of MAIT cells in *A. fumigatus* CEA10Δku80-infected mice could be seen from 4 DPI and reached a peak on 6 DPI (**Figure 6A**), corresponding with increased numbers of conventional T cells. By analyzing the pulmonary MAIT cells on 6 DPI, the absolute number of MAIT cells showed a dose-dependent increase (**Figure 6B**). At mid-range doses ($2.5x10^7$, $5x10^7$ CFU conidia), mice showed approximately 10^6 MAIT cells with nearly 100% survival rate. In repeat experiments, survival rate and weight change were found to be inconsistent due to gender and age of mice. Younger mice or female mice are more vulnerable as they have a lower starting weight (data not shown). Therefore, we set the infection dose for subsequent experiments to $3x10^7$ CFU of *A. fumigatus* CEA10Δku80 conidia to ensure that mice did not have severe weight loss or reach a humane endpoints during the experiment. This dose was suited to 6-7 weeks old male mice and 9-10 weeks old female mice with 100% survival rate and activation of pulmonary MAIT cells. Following the same procedure, the infection dose of *A. fumigatus* Af293 was determined at $1.5x10^7$ CFU.

To investigate the infection kinetics of *A. fumigatus* Af293, mice were infected intratracheally with $1.5x10^7$ CFU and MAIT cell and conventional T cell accumulation in the lungs was analysed following infection (2, 4, 6, 8, 12, 16 and 20 DPI) (**Figure 6C**). The accumulation of pulmonary MAIT cells in *A. fumigatus* Af293 infection was evident from 4 DPI and was accompanied by an increase of conventional T cells. MAIT cell numbers reached a high level 6 DPI (peaking 8 DPI), and remained at high levels until 20 DPI. In the following experiments, disease progression of *A. fumigatus* Af293 and *A. fumigatus* CEA10Δku80 strains were found distinct (as described in **2.2.2** and **4.2.1**). *A. fumigatus* Af293 ("inflammatory" strain) infection took longer to be cleared than *A. fumigatus* CEA10Δku80 ("hyper-inflammatory" strain), which was consistent with published descriptions [207]. This difference made it critical to understand the long-term changes of MAIT cell numbers and infection kinetics in *A. fumigatus* Af293 infection.

The MAIT cell percentage of total αβ T cells in *A. fumigatus* Af293 infection was much lower than in *A. fumigatus* CEA10Δku80 infection on 6 DPI (**Figure 7A**), while they had the same absolute numbers at the same time points (**Figure 7B**). This suggests more conventional T cell involvement in *A. fumigatus* Af293 infection on 6 DPI. As the "inflammatory" strain has a low clearance rate compared to the "hyper-inflammatory" strain *A. fumigatus* CEA10Δku80 [207], it can be hypothesized that this would induce a larger recruitment of conventional T cells.

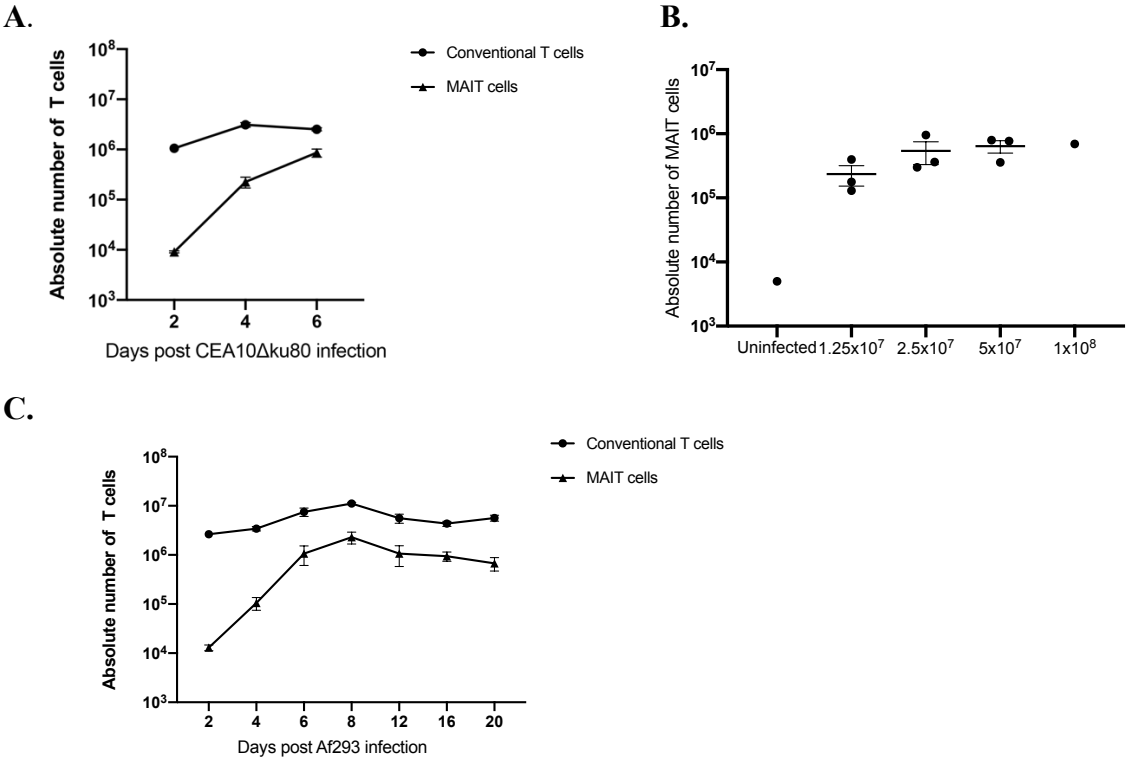


Figure 6. Accumulation of pulmonary MAIT cells and conventional T cells by i.t. infection with *A. fumigatus*

Kinetics of absolute numbers of MAIT cells and conventional T cells after infection with $3x10^7$ CFU *A. fumigatus* CEA10Δku80 (A) or $1.5x10^7$ CFU *A. fumigatus* Af293 (C). Experiments were performed in duplicate with similar results. n=5-6. mean ± SEM.

(B) Absolute numbers of pulmonary MAIT cells of C57BL/6 mice after intratracheal infection with different doses ($1.25x10^7$, $2.5x10^7$, $5x10^7$ or 10^8 CFU) of *A. fumigatus* CEA10Δku80 on 6 DPI. n=1-3. mean ± SEM.

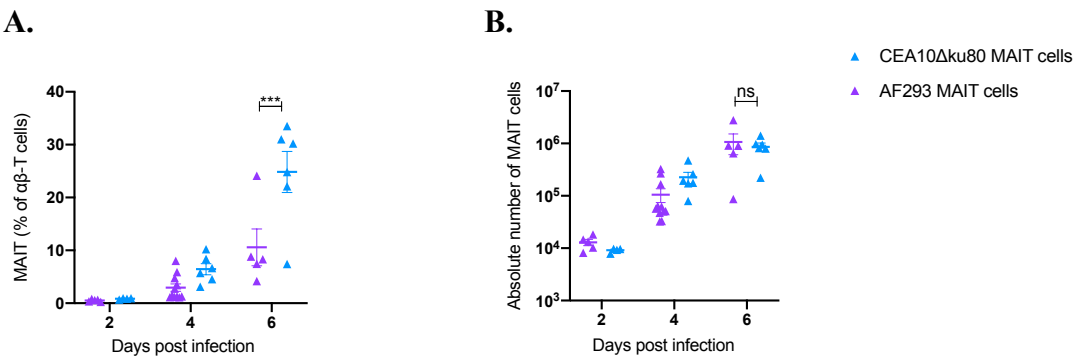


Figure 7. Accumulation of pulmonary MAIT cells by i.t. infection with two *A. fumigatus* strains

Kinetics of percentage (A) and absolute numbers (B) of MAIT cells after infection with $3x10^7$ CFU *A. fumigatus* CEA10Δku80 or $1.5x10^7$ CFU *A. fumigatus* Af293.

Experiments were performed in duplicate with similar results. n=5-6. mean ± SEM. ***P < 0.001, two-way ANOVA.

3.2.4 Accumulated MAIT cells display a MAIT-17 phenotype in early infection of *A. fumigatus*

Because the accumulation of MAIT cell driven by *A. fumigatus* was robust, it was necessary to assess their functional manifestations. To do that, the transcription factor expression pattern and cytokine production pattern of MAIT cells were analysed.

Transcription factors are the key to regulating gene expression and are closely related to cell differentiation and function. MAIT cells have been shown to express one of two transcription factors, T-bet and RORγt, which regulate differentiation. Using this method MAIT cells were described into two subsets in this study: MAIT-1 ($ROR\gamma t^{low}$ $T\text{-}bet^{hi}$) and MAIT-17 ($ROR\gamma t^{hi}$). Here, naïve mice displayed a predominant MAIT-17 phenotype in pulmonary MAIT cells, which was consistent with published data [141, 146]. The dominant MAIT-17 phenotype was also observed in MAIT cells in the lungs of mice that were infected with both *A. fumigatus* strains intratracheally, on 6 DPI (**Figure 8**).

The accumulated MAIT cells following *A. fumigatus* Af293 infection, showed a similar MAIT-17 phenotype on 6 DPI consistent with the *A. fumigatus* CEA10Δku80 intratracheally infected mice. However, as the infection progressed, a MAIT-1 population gradually appeared in *A. fumigatus* Af293-infected mice (**Figure 8B, C, D)**, while *A.*

fumigatus CEA10Δku80-infected mice still remained one main MAIT-17 cell subset on 10 DPI*[4] **(Figure 8A, C)**.

A previous study showed pulmonary MAIT cells frequencies can be enhanced by intranasal administration of 5-OP-RU plus TLR-2/9 agonists [221]. Another agonist of TLR-2, zymosan, was also found to enhance pulmonary MAIT cells frequencies with intranasal co-administration of 5-OP-RU by colleagues in the McCluskey Lab (data not shown). Zymosan is a ligand found on the surface of fungi, it has the possibility to represent a type of fungal infection. If the MAIT cells activated by zymosan plus 5-OP-RU display the same phenotype to *A. fumigatus* expanded MAIT cells, this method can be used as a vaccination to pre-boost MAIT cells before *A. fumigatus* infection. To assess this, C57BL/6 mice were administered zymosan (50 μg, resuspended with 0.02% Tween in PBS) intranasally and 5-OP-RU (76 pmol) on days 0, 1, 2, and 4, and pulmonary MAIT cells were analysed on day 6. Transcription factor analysis showed that MAIT cells display a MAIT-17 phenotype (**Figure 8E**). However, the effect of zymosan in boosting MAIT cell numbers was not consistent across all treated mice. In one group, some mice showed markedly increased MAIT cells, while others showed very few (data not shown), which made it an inappropriate way to pre-boost MAIT cells before the infection.

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*[4]Data of *A. fumigatus* CEA10Δku80-infected mice on 10 DPI were obtained in mice treated with neutrophil depletion before infection. Due to time constraints (COVID-19 lockdown), this was not performed with WT mice.

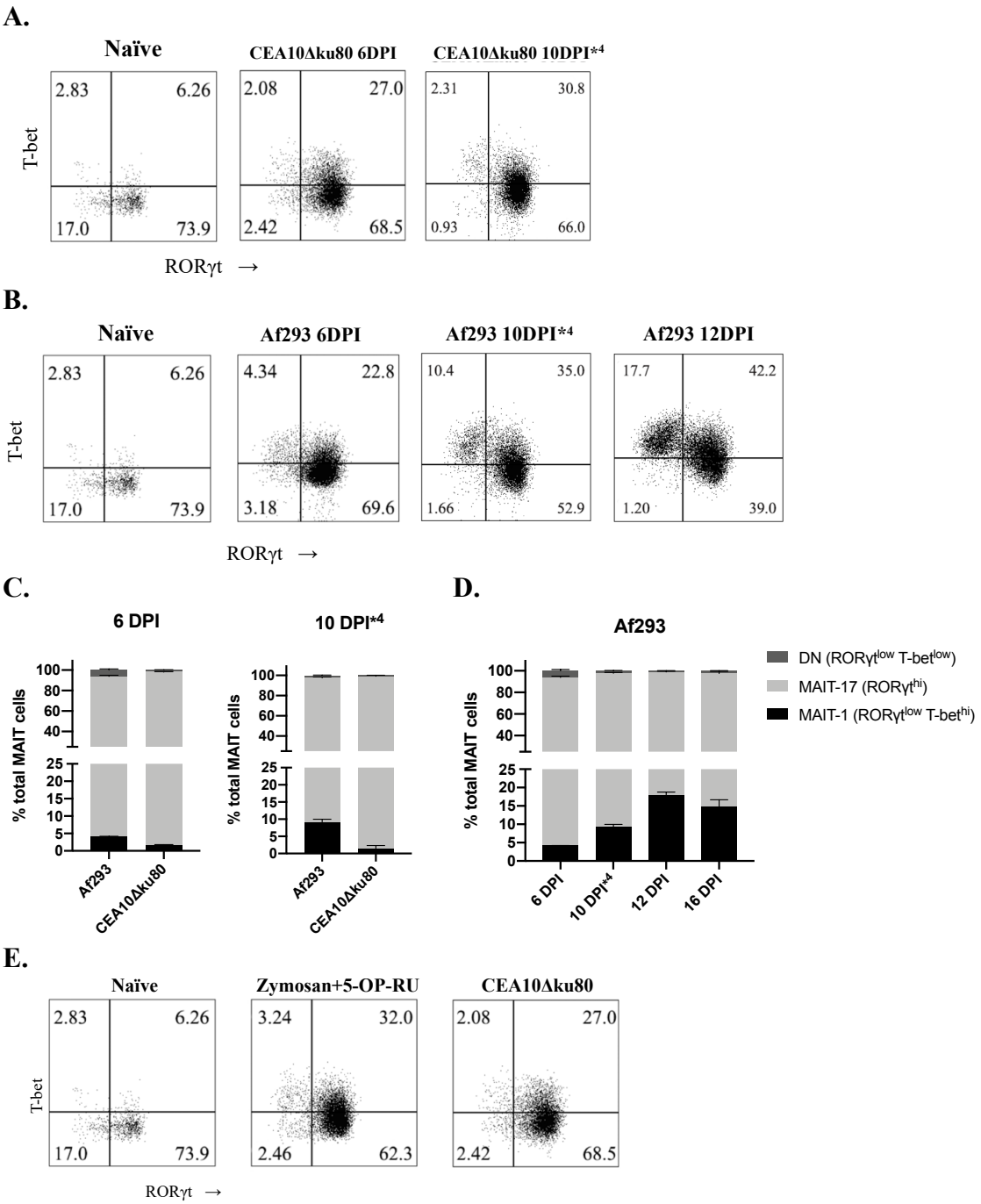


Figure 8. Transcription factor expression pattern of pulmonary MAIT cells in *A. fumigatus* infection

C57BL/6 mice were infected with $3x10^7$ CFU *A. fumigatus* CEA10Δku80, or $1.5x10^7$ CFU *A. fumigatus* Af293 intratracheally, pulmonary MAIT cells were analysed on different time points (6 DPI, 10 DPI, 12 DPI, and 16 DPI, as indicated)

Representative flow cytometry plots showing transcription factor staining of T-bet and RORγt of MAIT cells in C57BL/6 mice infected with *A. fumigatus* CEA10Δku80 (A), or *A. fumigatus* Af293 (B).

(C) Percentage of MAIT-1 (RORγt^{low} T-bet^{hi}), MAIT-17 (RORγt^{hi}), and double negative (DN, RORγt^{low} T-bet^{low}) population of total MAIT cells in the lungs of *A. fumigatus* CEA10Δku80 or *A. fumigatus* Af293 infected mice on 6 DPI and 10 DPI. n=3-4. mean ± SEM.

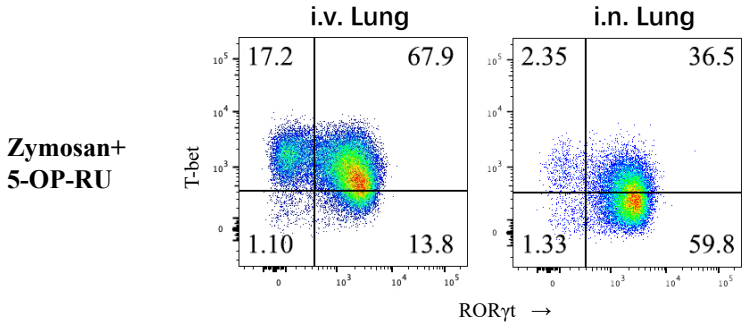
(D) Percentage of MAIT-1, MAIT-17, and DN population of total MAIT cells during *A. fumigatus* Af293 infection. Showing the gradually appeared MAIT-1 population as infection progressed. n=4-6. mean ± SEM.

(E) Mice were infected with 3x10^7 CFU *A. fumigatus* CEA10Δku80 or treated with zymosan (50 μg) plus 5-OP-RU (76 pmol) intranasally. Representative flow cytometry plots showing T-bet and RORγt expressing pattern in MAIT cells on 6 DPI.

Intravenous administration of zymosan also showed inconsistent activation of MAIT cells (data not shown). C57BL/6 mice were given zymosan (100 μg, resuspended with 0.02% Tween in PBS) intravenously, followed by intranasal administration of 5-OP-RU (76 pmol) on days 0, 1, 2, and 4. MAIT cells were analysed on day 6. In mice showing enhanced accumulation of MAIT cells, the transcription factor (T-bet and RORγt) expression pattern was different from zymosan intranasally treated mice (**Figure 9A**). With administration through the respiratory tract, activated MAIT cells in the lungs showed a MAIT-17 dominated phenotype, with few MAIT-1 cells observed. While with administration through the vein, pulmonary MAIT cells showed an increased frequency of the MAIT-1 population compared to naïve mice (**Figure 9A**).

The different expression pattern of T-bet and RORγt in MAIT cells with different administrations was also observed with *A. fumigatus* CEA10Δku80 infection (**Figure 9B**). However, the transcription factor staining of intravenous infected mice was only conducted once as the intravenous mode of inoculation was ceased early in the study.

A.



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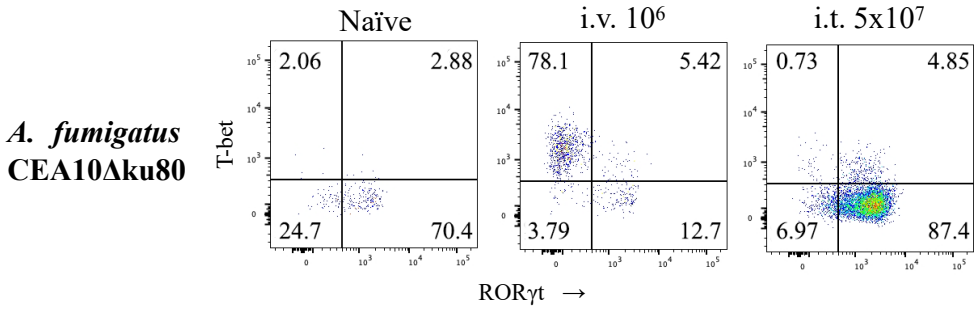


Figure 9. Different administration routes promote different MAIT cell transcription factor expression pattern.

(A) Representative flow cytometry plots showing transcription factor staining of T-bet and RORγt of MAIT cells in C57BL/6 mice treated with zymosan intravenously (100 µg) or intranasally (50 µg) with intranasal administration of 5-OP-RU (76 pmol) on days 0, 1, 2, and 4. MAIT cells of lungs were analysed on 6 DPI.

(B) Representative flow cytometry plots showing transcription factor staining of T-bet and RORγt of MAIT cells in C57BL/6 mice infected with *A. fumigatus* CEA10Δku80 intravenously (10^6 CFU conidia) or intratracheally ($5x10^7$ CFU conidia), MAIT cells of lungs were analysed on 6 DPI.

The transcription factor expression pattern reflect the functional phenotype of cells capable of producing different cytokines, which may have a different outcome of infection. Therefore to confirm that MAIT cells were producing Th1 or Th17 cytokines, cytokine production of accumulated MAIT cells was analysed by intracellular cytokine staining (ICS) and flow cytometry. Four cytokines that have been shown to be produced by MAIT cells [114, 117] were tested and compared production in the context of infection by the two strains of *A. fumigatus.* TNF and IFN-γ are typical cytokines secreted by Th1 cells, while IL-17A and GM-CSF are associated with Th17 function.

MAIT cells and conventional T cells were analysed on 6 DPI. At this time, MAIT cells represented 10% and 30% of pulmonary T cells in *A. fumigatus* Af293 and *A. fumigatus* CEA10Δku80 infected mice, respectively (**Figure 7A**).

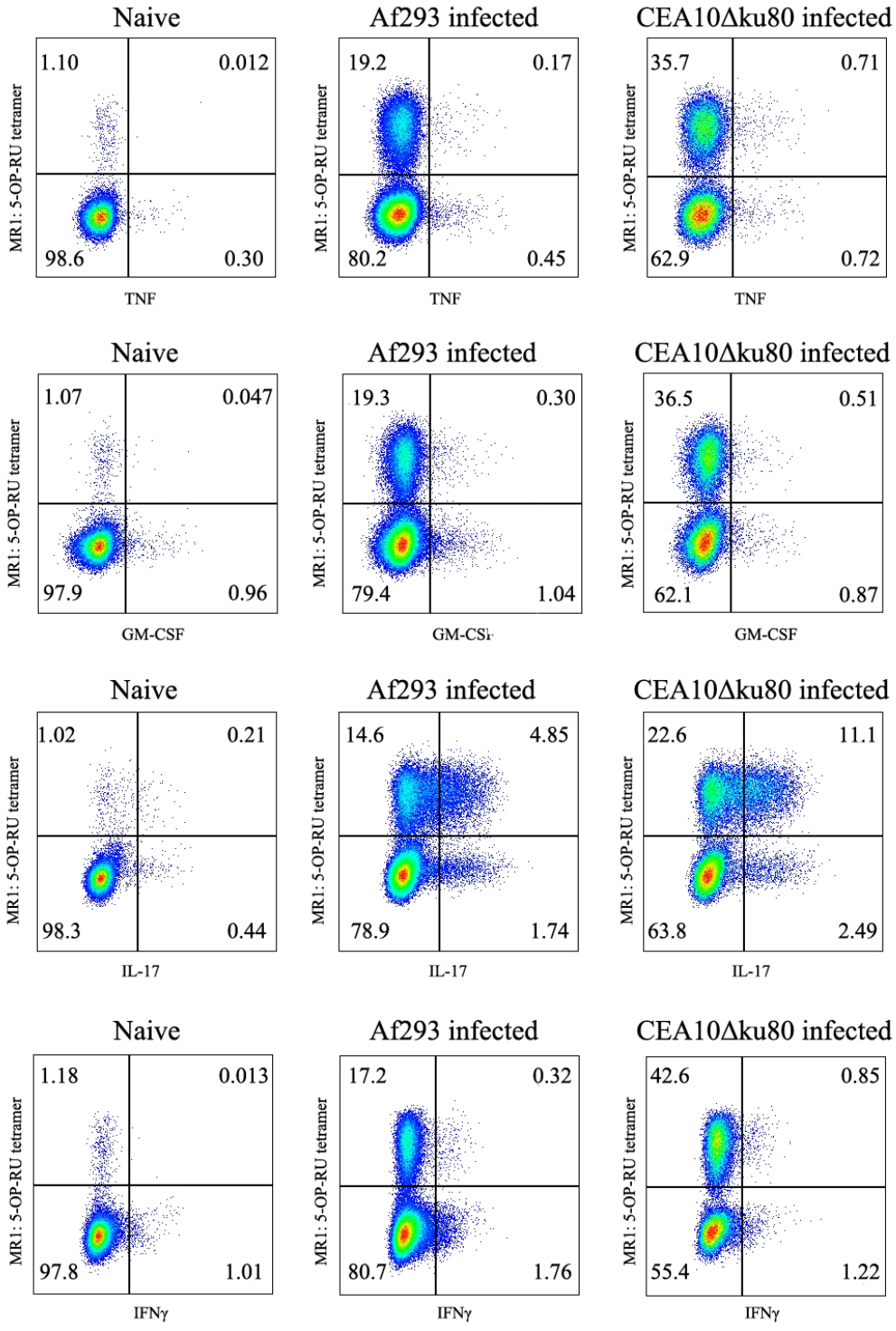
After infection with either *A. fumigatus* CEA10Δku80 or *A. fumigatus* Af293, IL-17A positive MAIT cells were significantly increased compared with naïve mice, which was consistent with the MAIT-17 phenotype defined by transcription factor staining (**Figure 10**).

The production of GM-CSF by MAIT cells was significantly decreased in infected mice compared to MAIT cells from naïve mice (**Figure 10**).

A. fumigatus CEA10Δku80 infected mice showed no significant increase in production of IFN-γ by MAIT cells compared to naïve mice. However, *A. fumigatus* Af293 induced MAIT cells showed an increased production of IFN-γ (**Figure 10B**). The higher production of IFN-γ could be a hint for the gradually appearing MAIT-1 population in *A. fumigatus* Af293 infection, which express Th1 function associated transcription factor T-bet (**Figure 8**).

TNF production was at same level in infected mice compared to the naïve state (**Figure 10**).

A.


Naive
Af293 infected
CEA10Δku80 infected
MR1: 5-OP-RU tetramer
TNF
1.10
0.012
98.6
0.30
19.2
0.17
80.2
0.45
35.7
0.71
62.9
0.72
GM-CSF
1.07
0.047
97.9
0.96
19.3
0.30
79.4
1.04
36.5
0.51
62.1
0.87
IL-17
1.02
0.21
98.3
0.44
14.6
4.85
78.9
1.74
22.6
11.1
63.8
2.49
IFNγ
1.18
0.013
97.8
1.01
17.2
0.32
80.7
1.76
42.6
0.85
55.4
1.22

B.

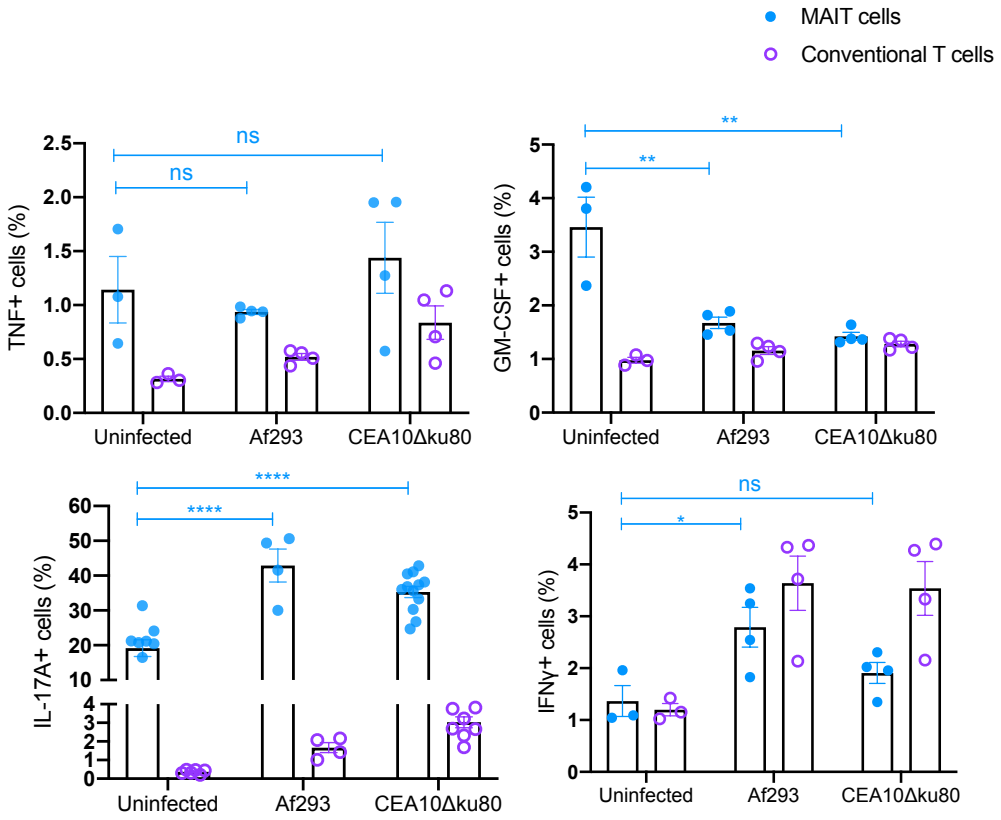


Figure 10. Cytokine profiles of pulmonary MAIT cells and conventional T cells in *A. fumigatus* intratracheal infection on 6 DPI

C57BL/6 mice were uninfected or infected with $3x10^7$ CFU *A. fumigatus* CEA10Δku80 or $1.5x10^7$ CFU *A. fumigatus* Af293 conidia intratracheally, cytokine production was analysed on 6 DPI by flow cytometry.

(A) Representative flow cytometry plots showing the intracellular staining for TNF, GM-CSF, IL-17A and IFN-γ of pulmonary TCRβ$^+$ T cells (non-MAIT T cells and MAIT cells) directly *ex vivo* from the lungs of mice with indicated treatment. Numbers represent the percentage of gated population of total TCRβ$^+$ T cells.

(B) Percentage of cytokine-expressing cells of MAIT cells and conventional T cells.

n=4-7, mean ± SEM. *P < 0.05, **P < 0.01, **** P < 0.0001. Ordinary one-way ANOVA.

3.2.5 The accumulation of MAIT cells in *A. fumigatus* CEA10Δku80 infection is IL-23-dependent

Typically, T cells require three signals for their activation: first is the binding of TCRs and MHC molecules presenting cognate antigen. The second signal is co-stimulation provided by cell surface molecules, such as between CD28 on T cells and CD80/86 protein on APCs. The third signal is cytokine stimulation; different cytokines will produce different responses. Wang, *et al.* demonstrated the accumulation and activation of MAIT-17 cells during pulmonary bacterial infection requires IL-23 [146]. To determine if the same requirements were needed for fungal infection, *Il-23p19*$^{-/-}$ mice were used in the context of *A. fumigatus* CEA10Δku80 infection. *Il-23p19*$^{-/-}$ mice lack the IL-23p19 unique subunit of IL-23 and therefore cannot produce IL-23 [146]. The MAIT cell number is slightly decreased in naïve *Il-23p19*$^{-/-}$ mice [222].

Il-23p19$^{-/-}$ mice received 3x10^{7} CFU *A. fumigatus* CEA10Δku80 conidia intratracheally and pulmonary MAIT cells were analysed on 6 DPI. Results showed the absolute number and percentage of MAIT cells was much lower than WT mice, suggested MAIT cell accumulation in *Il-23p19*$^{-/-}$ mice was impaired in *A. fumigatus* CEA10Δku80 infection. (**Figure 11**).

Because the majority of MAIT cells in the lungs of mice following *A. fumigatus* CEA10Δku80 infection was MAIT-17, which required IL-23 for the activation, the IL-23-dependent accumulation of MAIT cell was consistent with the MAIT-17 phenotypic characteristics in *A. fumigatus* CEA10Δku80 infection.

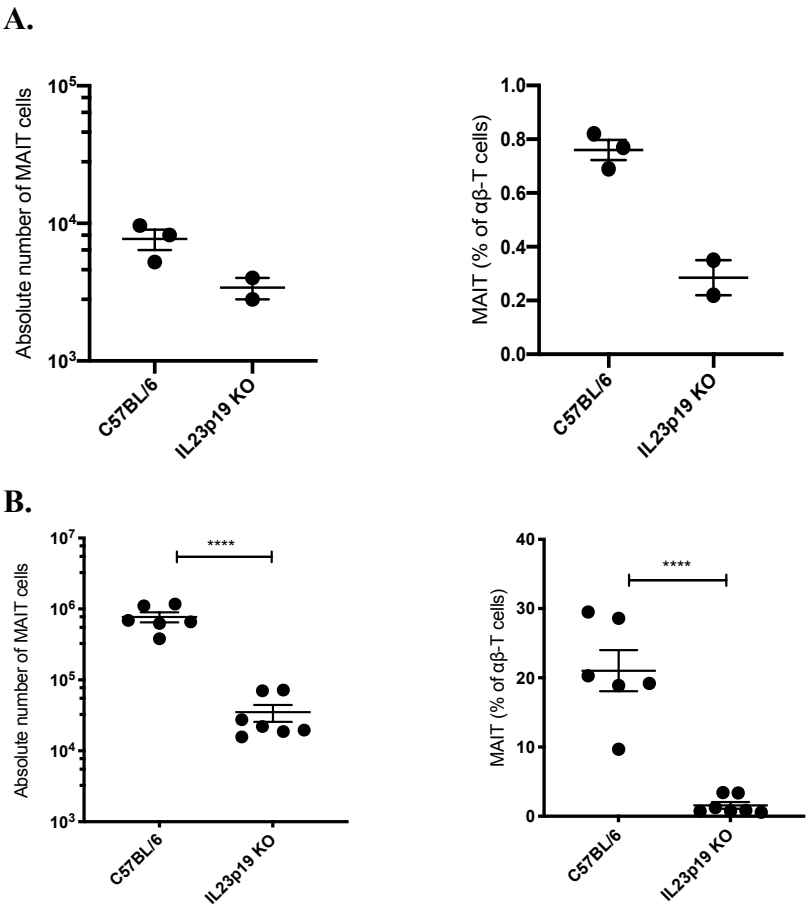


Figure 11. The accumulation of MAIT cells in *A. fumigatus* CEA10Δku80 infection is IL-23-dependent

Absolute numbers and percentage of MAIT cells isolated from lungs of uninfected WT mice and *Il-23p19*$^{-/-}$ mice (A), or infected intratracheally with 3x10^7 CFU *A. fumigatus* CEA10Δku80 on 6 DPI (B). means ± SEM, n=6-7, **** = P<0.0001, unpaired t test. Due to limited data, statistical test was not applied on (A).

3.3 Discussion

In this chapter, an *in vitro* assay was conducted to confirmed that *A. fumigatus* can activate MAIT cells. Supernatant from Aspergillus cultures activated Jurkat.MAIT cells as detected by the significant upregulation of CD69 expression, indicating the capability of *A. fumigatus* to produce MAIT cell activating antigens. However it was not clear as to why PDB culture failed to activate Jurkat.MAIT cells. This may because the complex components present in the PDB, which may inhibit MAIT cell activation or affect the growth of fungi. It illustrated the fact that the response of MAIT cells is affected by many factors, even *in vitro*.

To investigate MAIT cells during *A. fumigatus* infections *in vivo*, a mouse model with *A. fumigatus* intratracheal infection was established to assess MAIT cell response. Robust accumulation of pulmonary MAIT cells was observed in mice infected with both strains, with the increase of conventional T cell numbers. In order to pave the way for studying their function, the expression patterns of transcription factors of accumulated MAIT cells and their cytokine production were analysed. Accumulated MAIT cells in pulmonary infection with both *A. fumigatus* CEA10Δku80 and *A. fumigatus* Af293 showed a RORγt^{hi} MAIT-17 phenotype on 6 DPI, which was similar with the fungal ligand zymosan. However, further study showed that a MAIT-1 population gradually appeared in later *A. fumigatus* Af293 infection. It should be noted that, while MR1-dependent activation of Jurkat.MAIT reporter cells by *A. fumigatus* was confirmed *in vitro*, the requirement for MR1-TCR interaction *in vivo* was not tested in the current study. Analysis of cytokine profiles showed enhanced production of IL-17A on 6 DPI with both *A. fumigatus* CEA10Δku80 infection and *A. fumigatus* Af293 infection. Previous studies have demonstrated that IL-17A plays a role in Aspergillus infection [223-225]. Thus, MAIT cells may have a function in *A. fumigatus* infection as one source of IL-17A. However, the relative importance of other IL-17 producing cells has not been tested. As performed in a previous study in the context of *Legionella* infection [141], adoptive transfer of MAIT cells between IL-17 sufficient or deficient strains could be used to dissect their role as IL-17A producing cells.

In an *in vitro* study of human Aspergillus-specific T-cells, researchers found that stimulation with *A. fumigatus* proteins led to different phenotypes in lung-derived mononuclear cells (LMCs) and peripheral blood mononuclear cells (PBMCs). Aspergillus-specific T-cells in LMCs showed a Th17 cytokine profile with production of IL-17 and a limited production of IFN-γ, whereas Aspergillus-specific T-cells in PBMCs displayed a Th1 phenotype, producing mainly TNF and IFN-γ [224]. In the current study, it was found that different administration methods may affect the way MAIT cells acted. Following intratracheal infection, lung MAIT cells showed a MAIT-17 phenotype on 6 DPI, while intravenous infection led to both MAIT-1 and MAIT-17 phenotypes (**Figure 9A**). However, it is unknown whether this has any effect on the

outcome of infection by the different routes. This phenomenon also existed with zymosan administration (**Figure 9B**). Differences in cellular response dictated by different administration methods have also appeared in other studies. This may be related to the microenvironment the pathogen first encounters after entering the host. In the research of *F. tularensis* LVS, alveolar macrophages were found to be the first infected cells after intranasal inoculation, with a robust Th17 response on 7 DPI; while with intradermal inoculation, the bacteria need to disseminate from the skin to the lung, interstitial macrophages and neutrophils were found the dominant first infected cell types [226]. This may have an influence on the cytokine signal to adaptive immunity. Previous studies have shown that if IL-12 is present when the antigen is encountered, naïve $CD4^+$ T cells will differentiate into Th1 effector T cells, and if the cytokines are IL-6 and TGF-β, they will differentiate into Th17 effector T cell [227].

In previous bacterial infection studies, Wang, *et al.* demonstrated the accumulation and activation of MAIT-17 cells requires IL-23 [146]. They also found no significant effect of IL-23 deficiency on numbers of non-MAIT αβ T cells, NK T cells, γδ T cells, or ILCs in the lungs with *Legionella* infection [146]. In this study, the accumulation of MAIT cells was assessed only on 6 DPI, which was the peak of infection in WT mice. It is unclear whether IL-23 deficiency has impact on Aspergillus growth or the composition of other cell population. On 6 DPI, fewer MAIT cells were observed in *Il-23p19*$^{-/-}$ mice. The impaired accumulation of MAIT cells was consistent with the MAIT-17 phenotype they displayed. This experiment was not conducted with *A. fumigatus* Af293 due to time constraints. It is hypothesized the result may be different in *Il-23p19*$^{-/-}$ mice infected with *A. fumigatus* Af293 as a MAIT-1 population gradually appeared in the later stage of infection. In order to determine the impact of IL-23 deficiency during Aspergillus infection, further studies would include transcription factor staining and cytokine profiles analysis. This may show the role of IL-23 on the function mode of MAIT cells and/or other immune cells such as conventional T cells during infection. Also the cytokine production of MAIT cells in the later *A. fumigatus* Af293 infection was considered necessary in future studies, as the gradually appearing MAIT-1 population could affect the cytokine production pattern and may produce more Th1-type cytokines. It is not known why the initial IL-17-dominant response appeared to shift towards a IFN-γ later

on. It should be noted that the “hyper-inflammatory ” strain *A. fumigatus* CEA10Δku80 has a higher fungal growth in the lungs early in infection, and is cleared more quickly [207]. After 6 DPI, *A. fumigatus* CEA10Δku80 was nearly cleared in the lung, while *A. fumigatus* Af293 remained at more than 10^6 CFU (**Figure 12B**). Thus, it is hypothesized that different immune signals, such as a different cytokines, could drive MAIT cells into distinct functional phenotypes. However, it is not yet known if MAIT-1 and MAIT-17 populations are stable or can interconvert, or how long lasting this response would be. Therefore further studies are needed to assess the MAIT cell response with different fungal strains, throughout the course of infection.

In conclusion, *A. fumigatus* has been verified to activate MAIT cells *in vitro* and elicit pulmonary MAIT cell expansion in mice infected with conidia intratracheally. MAIT cell accumulation can be observed from 4 DPI and reached a relatively high level on 6 DPI (peaked on 8 DPI during Af293 infection). Both strains induce a robust expanded MAIT-17 population and large production of IL-17A on 6 DPI. Since early studies have demonstrated the more rapid fungal clearance of *A. fumigatus* CEA10Δku80 compared with *A. fumigatus* Af293 [207], the gradually appeared MAIT-1 population in a later *A. fumigatus* Af293 infection suggests that their function may change as the infection progresses. This suggests that MAIT cells play a role throughout *A. fumigatus* infection. However, more research is necessary to follow up the current study.

Chapter 4 Investigation of MAIT cell functions in *Aspergillus fumigatus* infection

4.1 Introduction

MAIT cells play a role in some pulmonary infections, including *Klebsiella pneumoniae* [154], *L. longbeachae* [141], *M. bovis* BCG [152], and *F. tularensis* LVS [144]. Most studies of MAIT cell functions in lung infections have assessed their role in response to bacteria. Considering the large proportion of MAIT cells in humans, and their dramatic activation during murine *A. fumigatus* infection, it was important to study the effect of MAIT cells on fungal infections.

As mentioned in Chapter 3, MAIT cells showed a significant response after *A. fumigatus* pulmonary infection, including a significant increase in cell number and cytokine production. Therefore, there was reason to believe that MAIT cells play a role in *A. fumigatus* infection. To examine the function of MAIT cells, fungal burden were compared between WT C57BL/6 mice and age- and gender-matched *Mr1*$^{-/-}$ mice (deficient in MAIT cells), and mice in which MAIT cells were first expanded with ligand and cytokine administration (MAIT cell pre-boosted mice).

4.2 Results

4.2.1 MAIT cell deficient *Mr1*$^{-/-}$ mice show potentially faster clearance of *A. fumigatus*

Mr1$^{-/-}$ mice lack the *Mr1* gene and thus, they cannot produce the MR1 molecule, which is required for successful development of MAIT cells [124]. Therefore, *Mr1*$^{-/-}$ mice are deficient in MAIT cells but other immune functions are intact, making them a good control to analyze MAIT cell functions.

To investigate the function of MAIT cells in *A. fumigatus* pulmonary infections, C57BL/6 mice and *Mr1*$^{-/-}$ mice were given *A. fumigatus* conidia intratracheally. Fungal burden was compared by CFU counts from homogenized lung tissue.

A. fumigatus CEA10Δku80 conidia were found to be cleared very quickly (**Figure 12A**). Within two days after infection, there were only 10^6 CFU detected from an infection dose of $3x10^7$. On 4 DPI, at which time the number of MAIT cells began to show significant increase, the fungal burden was already very low. On 6 DPI, only hundreds or thousands of conidia remained in the lung. This quick fungal clearance rate is consistent with published description of "hyper-inflammatory" strain [207].

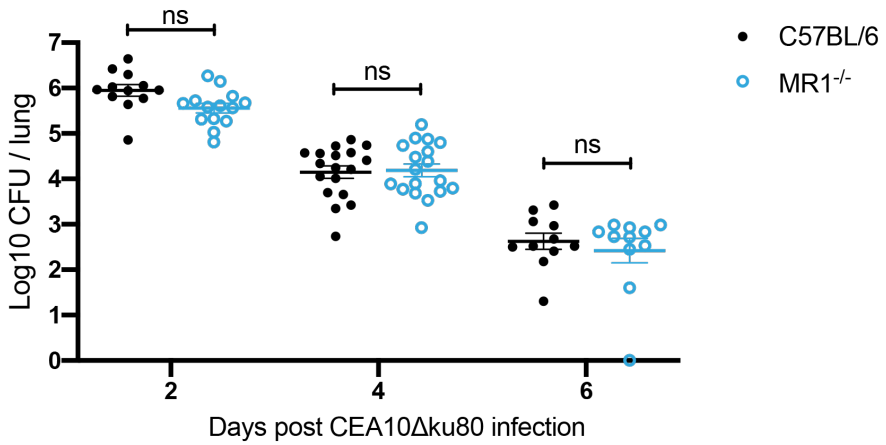
No differences were found between *Mr1*$^{-/-}$ mice and WT mice with *A. fumigatus* CEA10Δku80 infection (**Figure 12A**).

Due to the low fungal burden remaining on 6 DPI, it was decided not to continue with CFU counts at the later time points in *A. fumigatus* CEA10Δku80 infection.

Different from *A. fumigatus* CEA10Δku80, the clearance of *A. fumigatus* Af293 conidia was much slower (**Figure 12B**), most mice still had a high fungal burden (>10^6 CFU) even at 8 DPI. Thus more time points were included for *A. fumigatus* Af293 infection. The fungal clearance rate of *A. fumigatus* Af293 in each mouse strain appeared to be binary: some mice had a rapid fungal clearance (low CFU), while some mice remained a stable high level of fungal burden (high CFU). This indicated the large individual differences between mice. Although this may be due to inconsistent delivery of inoculum through technical issues, the difference was not observed before 8 DPI, suggesting this is not the explanation. Further experiments would be needed to determine the reason.

Even though when combining the results from all mice, *Mr1*$^{-/-}$ mice showed a significantly higher fungal clearance rate than WT mice on 20 DPI, the statistical difference was only observed when comparing one time point, hinting of the possibility of the false positive of the result of 20 DPI (**Figure 12B**). The exact reason for this contradiction needs further study in the future.

A.



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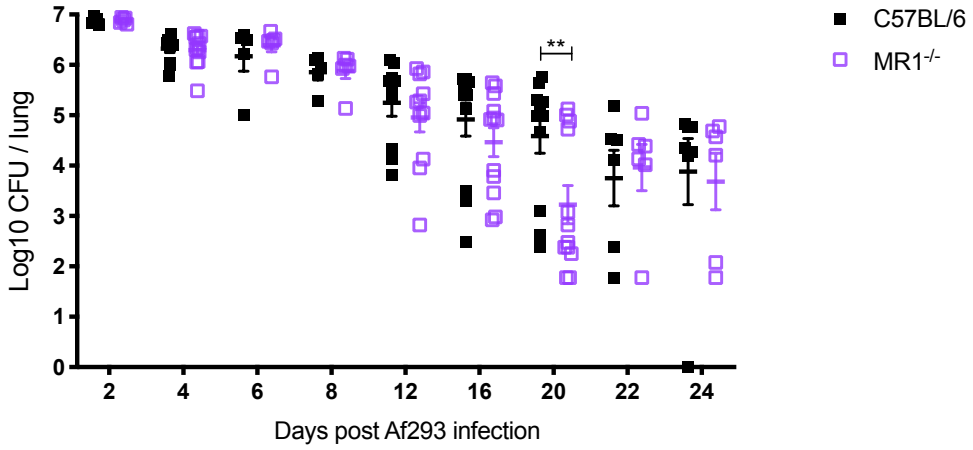


Figure 12. Fungal burdens during different strains of *A. fumigatus* pulmonary infection

Fungal load (CFU counts) in lungs of C57BL/6 or *Mr1*$^{-/-}$ mice at different time points after infection.

(A) Summary of the fungal load in lungs with 3x10^7 CFU *A. fumigatus* CEA10Δku80 conidia infection intratracheally, from four independent experiments. n=12 to 18.

(B) Summary of the fungal load in lungs with 1.5x10^7 CFU *A. fumigatus* Af293 conidia infection intratracheally, from two independent experiments. Due to COVID-19 lockdown, data of 22 DPI and 24 DPI were only from one experiment, and data of 20 DPI was composed of the data from 20 DPI and late 19 DPI. n=6 to 12.

Data show individual mice and means ± SEM, **P < 0.01, two-way ANOVA.

4.2.2 Immunocompromised mouse model for *A. fumigatus*

In experiments comparing the fungal burden in WT C57BL/6 and *Mr1*$^{-/-}$ mice, no clear difference was observed, except for a small significant difference at one-time point. Previous study showed MAIT cell function can be more pronounced in the absence of other immune cells [141]. Meanwhile, immune compromise also makes Aspergillus disease more evident, such as patients with neutropenia, organ-transfer, or treated with chemotherapy or radiotherapy, they cannot completely clear the conidia that have entered the body and the infection can develop into invasive disease [172]. Therefore, to investigate MAIT cell function in this condition, different immunocompromised mouse models were explored to assess the role of MAIT cells.

4.2.2.1 Chemotherapy agent treatment as a murine model for *A. fumigatus*

Chemotherapy is using anti-cancer medication to kill or damage cancer cells, but these agents also affect some healthy cells, in particular immune cells, causing side effects such as myelosuppression like leukocytopenia and thrombocytopenia [228]. Thus they can provide a model for immune suppression. In this study, two widely used chemotherapy agents were tried: Cortisone Acetate (CA), and Cyclophosphamide (CTX).

Cortisone is one of the glucocorticosteroid hormones which can be used as a treatment for a variety of cancers, such as leukemia, lymphoma, and multiple myeloma. CA can induce apoptosis in some cells (e.g., lymphocytes) [229]. CTX is a nitrogen mustard gas derivative that can be used in chemotherapy. CTX is inactive *in vitro* and becomes an active form after entering the body, and cause DNA damage during DNA replication.

For chemotherapy patients, the immune suppression caused by the agents impairs their immune cells and makes them more susceptible to Aspergillus infection [172]. The situation of this vulnerable group was simulated in mouse models by administering chemotherapy agents to mice.

For the initial attempt, a combined administration of the two agents was used. With reference to published data, CA (200 mg/kg) and CTX (150 mg/kg) were injected to mice subcutaneously and intraperitoneally respectively twice before infection (**Figure 13A**) [215, 218, 230].

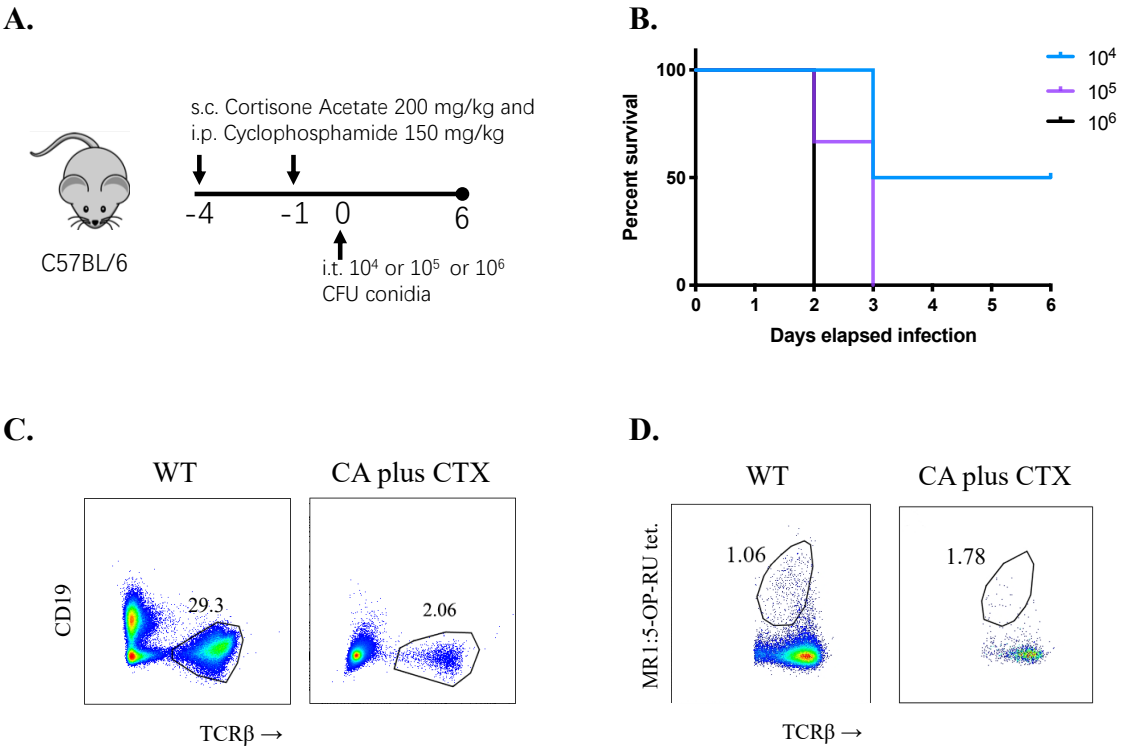


Figure 13. CA plus CTX-treated mice showed low populations of T cells and MAIT cells after *A. fumigatus* CEA10Δku80 infection

(A) Experiment scheme. C57BL/6 mice received CA (200 mg/kg, suspended in 0.02% Tween in PBS) subcutaneously and CTX (150 mg/kg) intraperitoneally on day -4, and day -1. 10^4, 10^5, or 10^6 CFU *A. fumigatus* CEA10Δku80 conidia were given intratracheally on day 0. Pulmonary cells were analysed on 6 DPI.

(B) Survival rate of combined agent treated mice after *A. fumigatus* CEA10Δku80 infection. For group 10^5, and 10^6, n=3; for group 10^4, n=4.

Flow cytometry plots of pulmonary TCRβ$^+$ lymphocytes (C) and MAIT cells (D) in one surviving mouse and WT mice (infected with 10^4 CFU *A. fumigatus* CEA10Δku80 conidia) on 6 DPI. Numbers indicate the percentages of TCRβ$^+$ lymphocytes (C) of total lymphocytes, and MAIT cells (D) of total TCRβ$^+$ lymphocytes.

Considering the immunosuppressive effect of chemotherapeutic agents, the optimal infectious dose for WT mice was expected to be too high for agent-treated mice. Therefore, 10^4, 10^5, and 10^6 CFU *A. fumigatus* CEA10Δku80 conidia were given to mice on day 0 (**Figure 13A**).

All of the CA plus CTX treated mice infected with 10^5 and 10^6 CFU conidia reached the humane end points within 3 days after infection, and only 50% of mice infected with 10^4 CFU survived until 6 DPI (end point of experiment) (**Figure 13B**). The pulmonary cells in these surviving mice were analysed by flow cytometry, and showed that the immunosuppressive effect of these agents in combination was so strong that mice had an only small population of TCRβ$^+$ lymphocytes (**Figure 13C**) and MAIT cells (**Figure 13D**). MAIT cell accumulation in Aspergillus infected mice is dose dependent (Figure 6B). However 10^4 CFU is the semi-lethal dose for CA plus CTX treated mice and due to ethical requirements, it was impossible to increase the infection dose to a dose which would be expected to induce higher MAIT cell numbers. Thus this model cannot obtain more MAIT cells and was replaced by single-agent administrations.

For single-agent administration, C57BL/6 mice were treated with CA (200 mg/kg) subcutaneously or CTX (150 mg/kg) intraperitoneally three times before infection with 10^5 CFU *A. fumigatus* CEA10Δku80 conidia intratracheally (**Figure 14A**).

In these two groups, all the mice survived to 6 DPI and lost less than 10% body weight with 10^5 CFU infection (**Figure 14B**). Absolute numbers of TCRβ$^+$ lymphocytes and MAIT cells in these mice were analysed on 6 DPI. In mice treated with CA only, the strong immunosuppressive effect on TCRβ$^+$ lymphocytes was observed (**Figure 14C**). Although the percentage of MAIT cells increased (**Figure 14D**), MAIT cell absolute numbers were very low level (**Figure 14C**), which made it an inappropriate model to study MAIT cell functions.

For the mice treated with CTX only, the results showed normal numbers of TCRβ$^+$ lymphocytes (**Figure 14C**). The lower weight loss, and the weight recovery that occurred before 6 DPI (**Figure 14B**) suggested that the low number of MAIT cells detected in the lungs at this time point may be due to the low dose of infection and thus a higher infection

dose may induce more MAIT cells. The model may have potential for further use to study MAIT cells if the inoculation dose can be increased to induce larger numbers. However, this model was not fully optimized and was suspended due to time constraints.

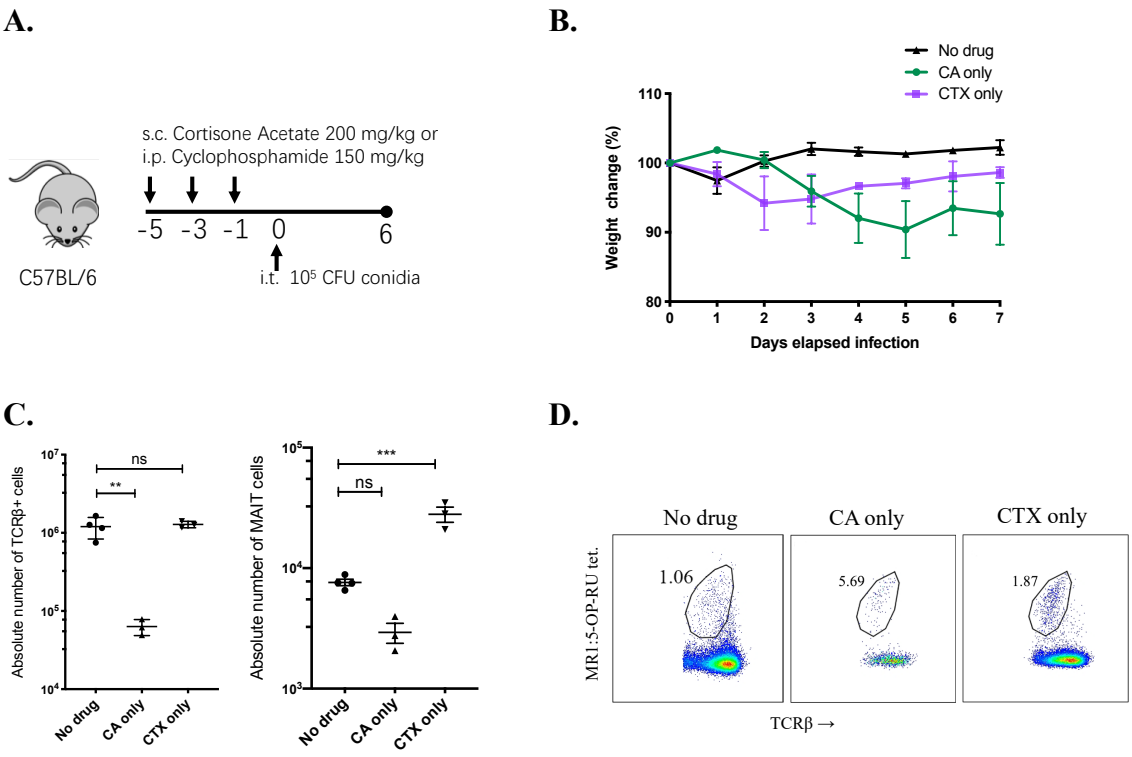


Figure 14. CA- or CTX-treated mice showed low populations of T cells or MAIT cells after *A. fumigatus* CEA10Δku80 infection

(A) Experiment scheme. C57BL/6 mice received CA (200 mg/kg, suspended in 0.02% Tween in PBS) subcutaneously or CTX (150 mg/kg) intraperitoneally on day -5, day -3, and day -1. 10^5 CFU *A. fumigatus* CEA10Δku80 conidia were given intratracheally on day 0. Pulmonary cells were analysed on 6 DPI.

(B) Body weight changes of 10^5 CFU-infected C57BL/6 mice treated with indicated agent. Data are presented as means ± SEM of mice per group, n=3.

(C) Absolute number of pulmonary TCRβ$^+$ lymphocytes and MAIT cells from 10^5 CFU-infected C57BL/6 mice treated with indicated agent (6 DPI). Data show individual mice and means ± SEM of 3 or 4 mice per group. **P < 0.01, ***P < 0.001, ordinary one-way ANOVA.

(D) Pulmonary MAIT cell percentage in 10^5 CFU-infected C57BL/6 mice treated with indicated agent (6 DPI). Numbers indicate percentages of MAIT cells of total TCRβ$^+$ T cells.

4.2.2.2 Neutropenic mouse model for *A. fumigatus*

Neutrophils are important innate immune cells and play a very important role in resisting Aspergillus infection [168, 185, 217]. They have the effect of removing Aspergillus conidia and hyphae, and can also mediate the elimination of Aspergillus by other cells [185]. Clinically, patients with neutropenia have an increased probability of infection with Aspergillus, and more easily develop invasive aspergillosis [172]. The high threat of Aspergillus infections they face makes it important to study the function of MAIT cells in a neutropenic model, which may provide a new idea for clinical treatment. In this study, anti-Ly6G antibody (mAb 1A8) was used to achieve a partial depletion of neutrophils in C57BL/6 mice, which mimics the situation of neutropenia patients. Ly6G is a cell surface marker expressed on monocytes, granulocytes and neutrophils. Although Ly6G is not strictly expressed only in neutrophils, using anti-Ly6G monoclonal antibody is currently the most widely used method to deplete neutrophils [231]. Studies have shown that this antibody does not bind to monocytes and macrophages [231]. Since there is no other appropriate alternatives at present, this is still a standard way to specifically deplete neutrophils.

To determine the dose of antibody, C57BL/6 mice were given anti-Ly6G antibodies (0.1 mg, 0.2 mg, or 0.5 mg) intraperitoneally and neutrophils were tested in the blood, lung and spleen by flow cytometry on the next day (**Figure 15**).

With 0.1 mg antibody, the population of neutrophils was significantly decreased compared with untreated mice on day 1. However, the depletion was incomplete and resulted in lower level of staining of Ly6G in all three organs in antibody-treated mice, especially in the spleen. This population did not disappear with increased amount of antibody (**Figure 15**). Consistent with these results, previous studies also demonstrated the incompleteness and dose independence of anti-Ly6G antibody depletion efficiency [232].

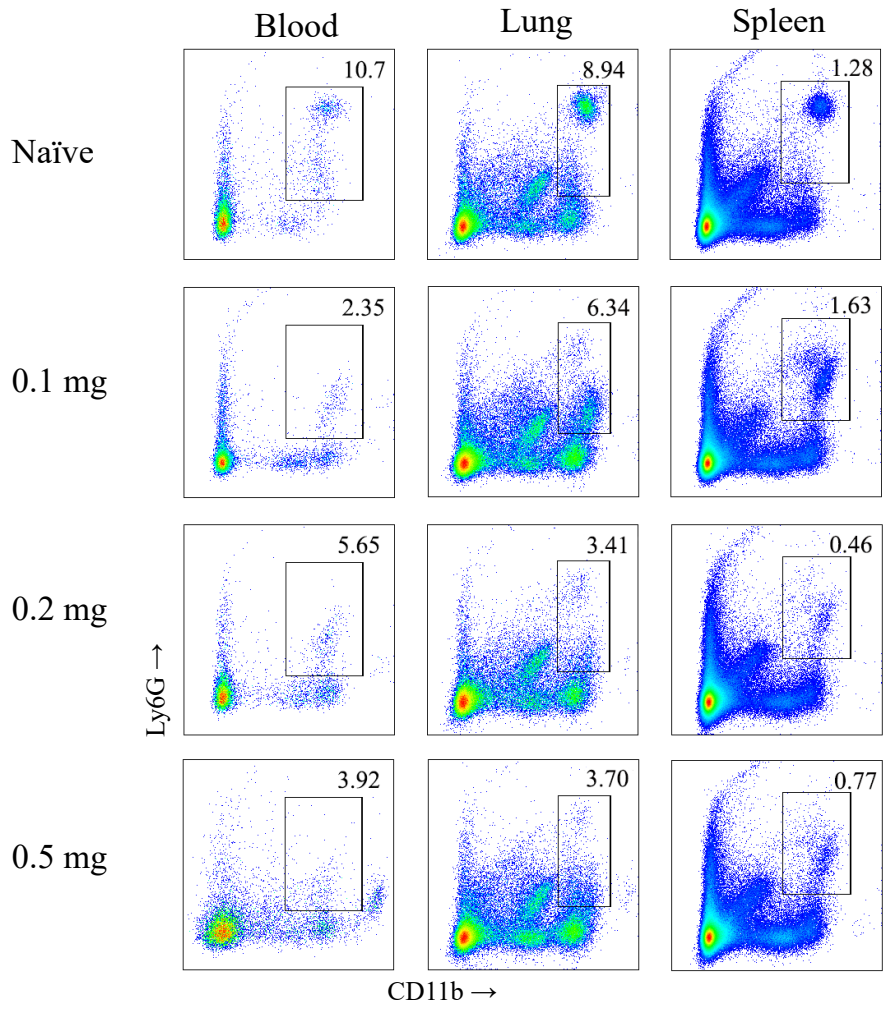


Figure 15. Neutrophil depletion effect of anti-Ly6G antibody with different dosage

Flow cytometry plots of live hematopoietic cells ($CD45.2^+$ $7\text{-}AAD^-$) showing $CD11b^+$ $Ly6G^+$ neutrophils (including the $CD11b^+$ $Ly6G^{low}$ population after anti-Ly6G antibody treatment) of three organs (as indicated) from C57BL/6 mice injected with 0.1 mg, 0.2 mg, or 0.5 mg monoclonal anti-Ly6G antibodies (mAb 1A8) intraperitoneally on day 1. Numbers show the percentage of neutrophils of total live hematopoietic cell.

A dose of 0.1 mg was chosen as the final dose for each injection. Mice were given 0.1 mg anti-Ly6G antibodies on the day before and after infection, and every 3 days after that (**Figure 16A**). The depletion effects during the experiments is shown in **Figure 16B**. Neutrophils were analysed using CD11b and Ly6G markers on day 0, day 3 and day 9 and no clear populations of neutrophils were observed (**Figure 16B**). However, a population with lower expression of Ly6G was seen at day 9.

These neutropenic mice were infected with different doses of *A. fumigatus* CEA10Δku80 (10^5, $3x10^5$, 10^6, 10^7, $2x10^7$ CFU) or *A. fumigatus* Af293 ($2.5x10^6$, $5x10^6$, 10^7 CFU) conidia intratracheally and the survival rate was measured.

In *A. fumigatus* CEA10Δku80 infection, mice infected with 10^7 or lower CFU conidia had an almost 100% survival rate (only one mouse infected with 10^5 CFU reached human endpoint on 8 DPI). With a dose of $2x10^7$ CFU (similar to the dose given to normal mice), there were still around 30% of mice surviving on 10 DPI (**Figure 16C**). This survival rate is much higher than the expected rate of mice in the invasive aspergillosis model [216], but lower than WT mice that were not treated with depleting Ab (**Figure 5B**), which had a 100% survival rate with dose below $5x10^7$.

In *A. fumigatus* Af293 infection, all the infected mice survived to 9 DPI even with the high infection dose of 10^7 CFU. The weight changes of these mice were shown in **Figure 16D**.

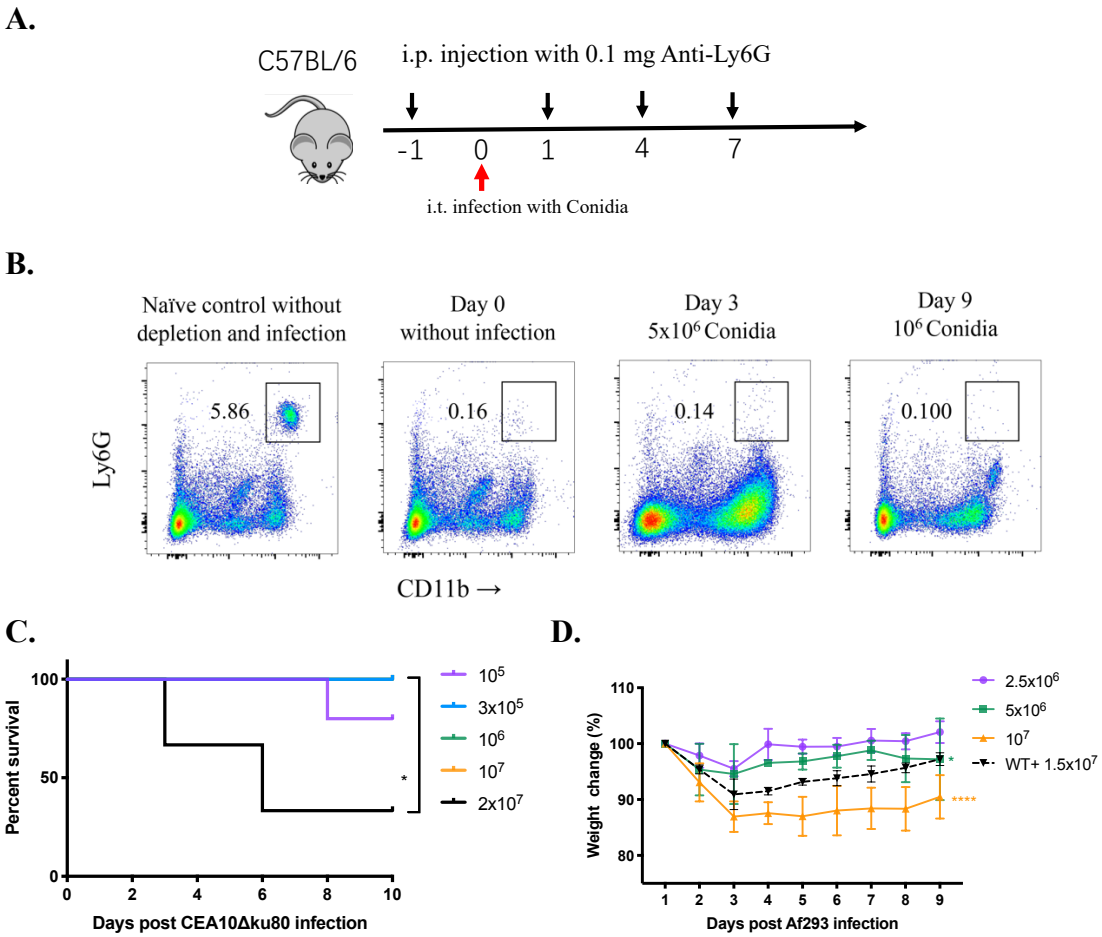


Figure 16. Neutropenic mice showed low resistance to *A. fumigatus*

(A) Experiment scheme. C57BL/6 mice received anti-Ly6G antibody (0.1 mg per injection) intraperitoneally on day -1, day 1, and every three days after that. *A. fumigatus* CEA10Δku80 (10^5, $3x10^5$, 10^6, 10^7, $2x10^7$ CFU) or *A. fumigatus* Af293 ($2.5x10^6$, $5x10^6$, 10^7 CFU) conidia were given intratracheally on day 0.

(B) The neutrophil depletion effect of anti-Ly6G during the experiment. Flow cytometry plots of $CD11b^+$ $Ly6G^+$ neutrophils (gated with black) in lungs in neutrophil depleted mice with/without infection. Numbers show the percentage of neutrophils of total lived hematopoietic cells ($CD45.2^+$ 7-AAD^-).

(C) Survival (defined as time to reach humane end points) of neutropenic mice infected with *A. fumigatus* CEA10Δku80. For group 10^7 and $2x10^7$, n=3; for group 10^5, $3x10^5$, and 10^6, n=5. (*P<0.05, compared with group $3x10^5$, which was 100% survival)

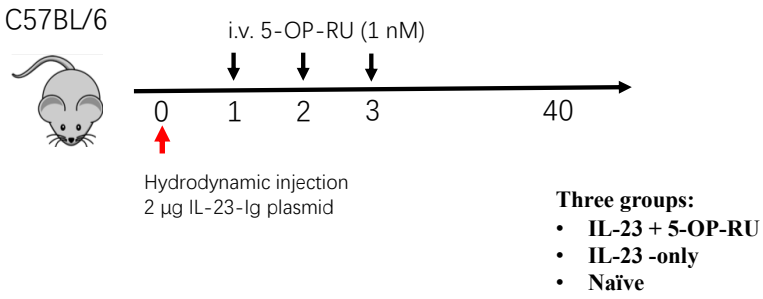
(D) Body weight changes of neutropenic mice and WT mice infected with *A. fumigatus* Af293. Data were presented as means ± SEM of mice per group (n=3 or 4). (*P<0.05, ****P<0.0001, compared with group $2.5x10^6$, two way ANOVA)

4.2.3 Pre-boosted MAIT cells show no significant effects on fungal clearance rate of *A. fumigatus* CEA10Δku80 pulmonary infection

Robust accumulation of MAIT cells suggested their high involvement during *A. fumigatus* infections. To assess MAIT cells' role, a method was conducted to boost MAIT cells prior to infection, and fungal burdens were compared between mice with/without pre-boosted MAIT cells. Previous work demonstrated the method to increase MAIT cell numbers before infection. By giving mice IL-23 plasmid by hydrodynamic injection and 5-OP-RU intravenously, MAIT cells can be enriched to 9% of total αβ T cells in the blood and around 30% of T cells in the lung on day 7 [146]. Boosted MAIT cells showed a MAIT-17 phenotype [146]. This method was used to demonstrate MAIT cell function in a study of *L. longbeachae*, in which mice were challenged with *L. longbeachae* 4-5 weeks later after IL-23 plasmid and 5-OP-RU treatment. Researchers found that mice with pre-boosted MAIT cells showed a faster pathogen clearance rate than untreated mice [146].

Here, C57BL/6 mice were given IL-23 plasmid by hydrodynamic injection on day 0. After this, 5-OP-RU was administered 3 times by intravenous injection on the following three days. Naïve mice and IL-23 plasmid-only administered mice were used as negative controls (**Figure 17A**). The MAIT cell percentages were analysed in these mice on day 40 (around 5 weeks later) to check the efficiency of MAIT cell pre-boosting. All mice were checked with blood samples and one mouse per group were culled to check the MAIT cells in the livers and lungs to confirm the results of blood testing (**Figure 17B**). The data showed that using the above method, boosted MAIT cells can persist over time. After 40 days, there were still around 10% of MAIT cells in the lungs (while in naïve mice MAIT cells only represent ~1% of pulmonary αβ T cells). All remaining mice from the three groups were infected on day 41. Fungal burden (CFU counts) in the lungs were analysed on 2 DPI, and 4 DPI (**Figure 18**).

A.



B.

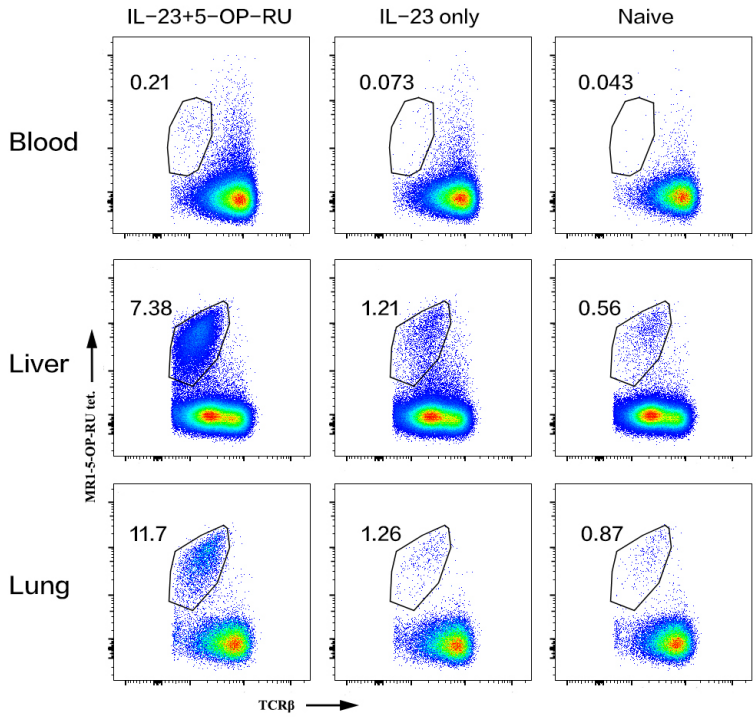


Figure 17. IL-23 plasmid and 5-OP-RU boosted MAIT cells can last for at least 40 days

(A) Experiment scheme. Three groups were set. All mice were age and gender-matched C57BL/6 mice. The naïve group was untreated. IL-23 only group received only IL-23-lg plasmid (2 μg) on day 0, while the IL-23+5-OP-RU group also received 5-OP-RU (200 μl, 1 nM) on day 1, day 2 , and day 3. MAIT cells in these mice were tested by flow cytometry on day 40.

(B) Flow cytometry plots of $TCR\beta^+$ T cells (with MAIT cells shown in gate) in the blood, livers and lungs. Numbers indicate MAIT cell percentages of total $TCR\beta^+$ T cells.

The results of fungal burden showed no difference between MAIT cell pre-boosted mice and un-boosted mice following infection with *A. fumigatus* CEA10Δku80 (**Figure 18**), suggesting that increased numbers of MAIT cells had no significant effect on the clearance rate of *A. fumigatus* CEA10Δku80.

Due to time constraints, *A. fumigatus* Af293 infection was not conducted in this pre-boosted MAIT cell model.

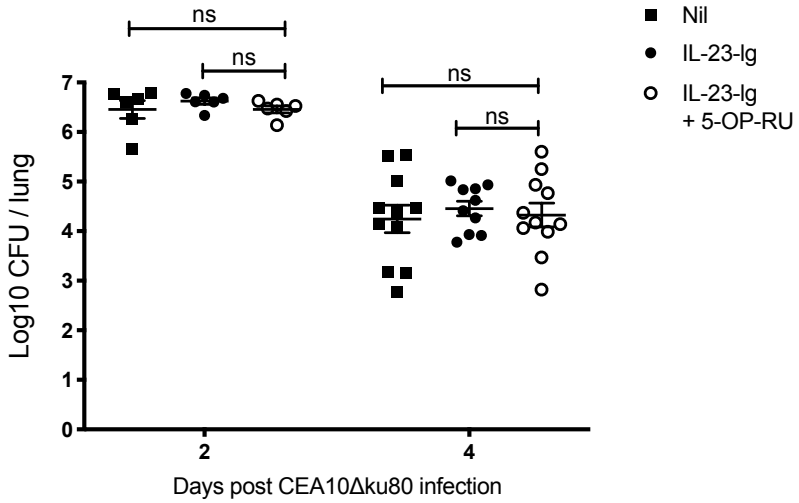


Figure 18. Pre-boosted MAIT cells show no significant effects on the fungal clearance rate during *A. fumigatus* CEA10Δku80 infection

Fungal load (CFU counts) in lungs of naïve C57BL/6 mice, IL-23-treated mice, or IL-23+5-OP-RU-treated mice (given IL-23-lg intravenously, and 5-OP-RU intranasally at following 3 days, infected mice after 40 days) at 2 DPI, and 4 DPI with $3x10^7$ CFU *A. fumigatus* CEA10Δku80 conidia intratracheally, from two independent experiments. Data show individual mice and means ± SEM, n= 6 to 11, two way ANOVA.

4.3 Discussion

The robust accumulation and cytokine production shown in Chapter 3 indicated a possible function of MAIT cells in protecting against *A. fumigatus* infection. To investigate this, the fungal burden in WT mice and *Mr1*$^{-/-}$ mice as well as mice with pre-boosted MAIT cells were analysed in this Chapter. Several immunodeficient murine models were studied with *A. fumigatus* infection.

In *A. fumigatus* Af293 infection, a difference of fungal burden between WT mice and *Mr1*$^{-/-}$ mice were observed on 20 DPI. However this needs further confirmation. Note that the fungal burden in each mouse group were binary, meaning that differences within the group were large. The unique difference shown on 20 DPI may due to sampling error, which happened to exist in both independent experiments. Recently, there has been increasing evidence that microbiota has impact on host immunity [233, 234]. However, it is still unclear whether there are differences in microbiota between C57BL/6 mice and *Mr1*$^{-/-}$ mice. Further studies are needed for more understanding of the immunologic mechanism.

It was considered the difference between WT mice and *Mr1*$^{-/-}$ mice during *A. fumigatus* Af293 needs to be confirmed before testing the effect of boosting MAIT cells for this infection. Although 5-OP-RU in the presence of zymosan did not result in consistent boosting of MAIT cells (Chapter 3), other methods of pre-boosting MAIT cells such as 5-OP-RU and TLR agonist administration [117] also should be considered due to the possibility that different boosted MAIT cells may give a different result.

For the immunodeficient murine models, it was attempted to develop a model in which other immune cells were impaired such that the role of MAIT cells could be better observed. CA and CTX were chosen due to their wide usage in the clinic as chemotherapy agents which suppress the immune response. However, the CA treatment caused the loss of TCRβ^+ lymphocytes including MAIT cells, and this low number of MAIT cells made it an inappropriate model to study their function. The CTX - model showed more promise, but further optimization of agent dose and infection dose is needed. For the neutropenia model, this results were inconsistent with published data. Further optimisation could include increasing mAb dose or the use of different mAbs for depletion as outlined below. For the neutropenia model, inconsistency of survival rate was observed with published data, the possible reason may be the technical issues during the neutrophil depletion. From a published study in 2014, researchers found a 80% and 100% mortality rate with $2x10^6$ and $5x10^6$ CFU *A. fumigatus* Af293 pulmonary infection respectively within 8 days in neutropenic mice. They used BALB/c mice and administered 0.5 mg anti-Ly6G (1A8) one day before and after infection [216]. Therefore it is possible that the lower antibody dose used here, and mouse strain differences could explain the different results. Another

issue is that the same marker (Ly6G) was used for both detection and depletion of the neutrophils [232]. In theory, this would not affect the depletion, but it would affect the accuracy of detection. Thus, future studies could include enumerating neutrophils after depletion with a different method, such as detecting neutrophils as $GR1^{int/\ hi}$ $Ly6C^{int}$ $CD11b^{+}$ cells [232], or other depletion methods such as RB6-8C5 monoclonal antibody which reacts with mouse Gr-1 [217] could be introduced to ensure the depletion efficiency of neutrophils. Once this model is further optimised future experiments could test whether the apparent dose dependent survival of infected neutropenic mice could be altered by boosting of MAIT cell responses.

In conclusion, although some fungal clearance differences were observed between WT mice and *Mr1*$^{-/-}$ mice during infection with *A. fumigatus* CEA10Δku80 and *A. fumigatus* Af293, no clear conclusion can be drawn at this time. Maybe fungal burden (CFU counts) is too blunt to reveal MAIT cell functions. Other readouts have the possibility to show the role of MAIT cells more clearly, such as Grocott's methenamine silver (GMS) stain which specifically shows the fungus, making hyphae invasion through the lung tissue to be visualized [235]; or qPCR which allows a more accurate assessment of fungal burden. Besides directly acting on the clearance of pathogen, MAIT cells could have other detrimental functions such as causing pathology in infection or could be important for tissue repair, both of which have been shown in many different settings [136]. It is worthwhile to assess these functions in this *A. fumigatus* model. Methods such as Hematoxylin and Eosin (HE) staining that shows the tissue condition [235] could be included in further study to study the consequences of MAIT cell responses to infection.

Chapter 5 General discussion

Aspergillus species are very common saprophytic fungi, which can grow almost everywhere [163]. Conidia of Aspergillus are present in the air in large amounts, and individuals can inhale hundreds of conidia every day [167, 168]. Although healthy people have the ability to completely remove inhaled conidia, people with certain diseases (such as those causing immunodeficiencies) face a serious threat from Aspergillus infections [172]. MAIT cells are innate-like T lymphocytes; unlike conventional T cells which recognize protein ligands, MAIT cells can be activated by vitamin B metabolites derived from the riboflavin biosynthetic pathway [132]. This metabolic pathway exists in many bacteria and fungi [134], including *A. fumigatus*. In 2018, a study demonstrated human MAIT cells can be activated by *A. fumigatus in vitro* [162], which provided a new consideration about the possibility of MAIT cells in Aspergillus disease treatment. Many previous studies have revealed the function of MAIT cells in bacterial infections [141, 144, 152, 154] in animal models, but to the best of the author's knowledge, the study discussed in this thesis is the first one assessing MAIT cell responses in Aspergillus infection *in vivo*. Here, a mouse model with *A. fumigatus* intratracheal infection was established to study MAIT cell function. In the complex *in vivo* environment, the proliferation, population characteristics and cytokine production patterns of MAIT cells were investigated during Aspergillus infection in Chapter 3, and the function of these accumulated MAIT cells were explored in Chapter 4. The fungal clearance rate were compared between WT mice, *Mr1*$^{-/-}$ mice, and mice with pre-boosted MAIT cells; and the infection process in immunodeficiency mice were discussed.

At the same time, there were some limitations in this study. Although many detailed data on the nature of MAIT cells during *A. fumigatus* infection were obtained, no clear conclusion about the role of these cells can be drawn currently. The limitations and implications of this study are discussed in this Chapter.

5.1 Dramatic activation and expansion of MAIT cells driven by *Aspergillus fumigatus* infection

MAIT cells are innate-like T lymphocytes which are found in high frequency in human peripheral blood and other organs including the gastrointestinal tract, liver and lung [119-122]. However, the frequency of MAIT cells is very low in common laboratory mouse strains housed under SPF conditions [114]. Previous studies which have shown a role of MAIT cells *in vivo* all demonstrated a striking accumulation of MAIT cells after infection. For example, in a study of *F. tularensis* LVS pulmonary infection, Meierovics, *et al*. described a large MAIT cell accumulation by enumeration of $CD4^-$ $CD8^-$ αβ T cells and assessing Vα19-Jα33 transcripts [144]; another study that revealed the protective function of MAIT cells during *L. longbeachae* lung infection described a 580-fold increase of MAIT cells, detected by MR1:5-OP-RU tetramer straining, compared with naïve mice [141].

Here, the dramatic expansion was also observed after *A. fumigatus* intratracheal infection. More than 10^6 pulmonary MAIT cells were found on 6 DPI with both *A. fumigatus* CEA10Δku80 and *A. fumigatus* Af293 intratracheal infection (**Figure 6**), close to the peak number of MAIT cells in the *L. longbeachae* study [141]. This large accumulation of MAIT cells showed their high involvement in immune responses during infections and suggested an important function in this setting.

5.2 Phenotype of pulmonary MAIT cells in intratracheal infection

In naïve MAIT cells, transcription factor expression pattern is mainly composed of the MAIT-17 ($ROR\gamma t^{hi}$) phenotype. Unlike the *in vitro* assay, MAIT cells cannot be activated by 5-OP-RU antigen alone *in vivo* [117]. They need other costimulatory signals to help in the process of activation, such as ICOS, and cytokines [146]. The expansion of MAIT cells has been demonstrated with TLR agonists or certain cytokines such as IL-23 delivered with 5-OP-RU [146, 221].

Pathogens that can activate MAIT cells provide sufficient signals including ligands and cytokines [117]. During the infection, MAIT cells will proliferate and the expression pattern of transcription factors will change according to the pathogen and administration

methods [146, 226], which may cause a different local immune environment. Previous studies showed, with intranasal administration, MAIT cells driven by *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) infection displayed a MAIT-17 dominated phenotype (>91%), while in *L. longbeachae* infection, the dominance of MAIT-17 was less marked, with a 10% MAIT-1 (RORγt^{low} T-bet^{hi}) population appearing on 7 DPI [141].

In the early stages of *A. fumigatus* infection, MAIT cells from both *A. fumigatus* Af293 and *A. fumigatus* CEA10Δku80 infected mice showed the same transcription factor expression pattern; most MAIT cells showed a MAIT-17 phenotype (>91%). However, in the later stages, *A. fumigatus* Af293 infected mice showed a small population of MAIT-1 cells (>15%) on 12 DPI. This pattern transition found in *A. fumigatus* Af293 infection suggested the changes in the immune environment of mice, such as condition of other immune cells and the cytokine level, and also suggested that as the infection progresses, the role of MAIT cells had changed.

5.3 The potential function of MAIT cells in *Aspergillus fumigatus* infections

The results of intracellular cytokine staining suggested a large production of IL-17A by MAIT cells in the early stage of *A. fumigatus* infection. Many researchers have demonstrated the importance of IL-17 in Aspergillus infection [119, 223-225], but the effect of this cytokine and its functions among different *A. fumigatus* strains are poorly understood. The production of cytokine in airway (bronchoalveolar fluid) or pulmonary tissue could be measured by enzyme linked immunosorbent assay (ELISA) or cytometric bead array (CBA). Here, the assessment of potential differences in fungal burden between WT mice and *Mr1*$^{-/-}$ mice needs further confirmation. For the purposes of this discussion it is assumed the results obtained were true positives.

In a study assessing eosinophils in aspergillosis, Guerra, *et al*. found that eosinophils are an important source of IL-17, and challenge with *A. fumigatus* CEA10 conidia led to a significantly higher mortality rate in ΔdblGATA-1 mice (deficient in eosinophils) compared with WT BALB/c mice, with a paradoxically decreased number of fungal burden (CFU counts) in the lung in ΔdblGATA-1 mice on 2 DPI. The difference in mortality rate made them believe the protective role of eosinophils via IL-17 during *A.*

fumigatus pulmonary infection [223]. In the study of this thesis, no difference of fungal burden (CFU counts) in the lung was observed in *Mr1*$^{-/-}$ mice on 2 DPI with *A. fumigatus* CEA10Δku80 infection (**Figure 11A**).

Guerra, *et al*. also used *A. fumigatus* Af293 in their study to investigate the role of eosinophils, but they did not observe any difference between mice with or without eosinophils using this strain [223]. Published data had demonstrated that *A. fumigatus* CEA10 is more virulent in acute infection than *A. fumigatus* Af293 [207]. It is therefore perhaps that the innate immune cells eosinophils which respond quickly to pathogens showed obvious importance during *A. fumigatus* CEA10 but not *A. fumigatus* Af293 infection. The results of this study showed that the fungal burden (CFU counts) of *Mr1*$^{-/-}$ mice was lower than WT C57BL/6 mice on 20 DPI in *A. fumigatus* Af293 infection (**Figure 11B**). Because of the paradox described by Guerra, *et al*, the lower fungal burden found in *Mr1*$^{-/-}$ mice was not enough to deny a potential positive effect of MAIT cells [223]. The reduced pathogen clearance rate caused by MAIT cells could be a positive effect which protected the tissue from excessive damage by reducing the immune inflammatory response. Previous studies discovered a Tc17 subset in mice which coupled antimicrobial and tissue repair functions [236, 237]. The type-17 phenotype was also found in MAIT cells in early *A. fumigatus* infection. Researchers demonstrated some tissue repair-related genes were significant enriched in TCR-dependent MAIT cell stimulation, including genes of TNF, Furin, CSF1, CCL3 and others, suggested MAIT cell function in not only antimicrobial but also tissue repair [136, 221, 222]. In this study, production of the cytokines TNF, IFN-γ, IL-17A and GM-CSF were analysed by intracellular cytokine staining on 6 DPI. Although a significant upregulation of IL-17A production was observed with both *A. fumigatus* CEA10Δku80 infection and *A. fumigatus* Af293 infection, the effect of this is unclear. It is possible that IL-17A has a role in MAIT cell-mediated tissue repair during *A. fumigatus* pulmonary infection, such as helping with epithelial barrier repair. Besides these four cytokine tested, analysis of other cytokines or factors during *A. fumigatus* infection is also needed to understand MAIT cell functions, for example IL-22, which made by MAIT cells [118] and strongly affects epithelial cell function [238]; and factors such as Furin, CSF1, CCL3, which are found enriched during MAIT cell activation [136].

Besides the evidence which supports a protective function of IL-17, another view is that IL-17 has a negative function which contributes to pulmonary injury during *A. fumigatus* infection. This is supported by the research of Malacco, *et al.* who used ΔdblGATA-1 mice infected with a CEA17-derived A1163 strain of *A. fumigatus*. This study showed that IL-17 led to more severe lung dysfunction and higher mortality rate; this phenomenon could be reversed by anti-IL-17 treatment [225].

These two studies by Guerra, *et al* and Malacco, *et al.* indicated different roles of IL-17 in *A. fumigatu*s infection. The host response induced by IL-17 can help with anti-pathogen responses, and also can cause tissue damage. As a source of large amounts of IL-17A, MAIT cells may also have a role in these two functions.

In this study, accumulated MAIT cells in *A. fumigatu*s Af293 infection produced large amounts of IL-17A in early infection. However, the presence or absence of MAIT cells did not make a difference in early fungal burden (CFU counts) or mortality. The lower fungal burden observed in *Mr1*$^{-/-}$ mice on 20 DPI suggested the transition of MAIT cell phenotype made its function more obvious, which may be achieved by changes in the cytokine production pattern. Therefore, it is necessary to analyse the cytokine pattern in later infection. The gradually appeared MAIT-1 population in *A. fumigatu*s Af293 infection could bring more Th1 type cytokines, such as IFN-γ. Previous studies showed IFN-γ treatment could significantly reduce the mortality of Aspergillus-infected CA-treated mice [239]. Decreased incidences of invasive aspergillosis were also observed in clinical patients who received IFN-γ [240]. These all indicated the protective role of IFN-γ in Aspergillus infections. As the intracellular cytokine staining already showed hints of increased IFN-γ production of MAIT cells on 6 DPI, it is reasonable to suggest that MAIT cells will produce more IFN-γ and participate in the response against Aspergillus at the late stage of infection.

Currently, fungal burden (CFU count) and mortality were used to measure the infection status, further study will include other readouts which reveal the condition of pathology, recruitment of other cells, tissue damage, or invasive disease progression, such as GMS stain which specifically shows the fungal morphology and can be used to assess the invasion [235], or HE stain which shows the tissue condition [235]. In a study of

Helicobacter pylori infection, D'Souza, *et al.* demonstrated MAIT cell function in accelerating gastritis by comparing the HE-stained stomach sections between WT mice and *Mr1*$^{-/-}$ mice. Fewer neutrophils, macrophages, eosinophils and DCs were observed in *Mr1*$^{-/-}$ than WT mice following infection, suggesting MAIT cell function in other immune cell recruitment [241]. This may also be present in Aspergillus infections, and warrants further investigation.

In summary, current data indicated that MAIT cells are involved in the immune process of *A. fumigatus* infection, as demonstrated by their dramatic accumulation and cytokine production, but no clear conclusion about their specific role in either protecting against infection or mediating pathology can be drawn based on current understanding.

5.4 Conclusion of the thesis

In conclusion, the study presented in this thesis first demonstrate the establishment of an *A. fumigatus* intratracheal infection mouse model to study MAIT cells *in vivo*, and the robust MAIT cell accumulation in this model. Furthermore, we observed a Th17-like phenotype (MAIT-17) in early infection of both *A. fumigatus* CEA10Δku80 and *A. fumigatus* Af293, and the gradual appearance of a MAIT-1 population, which was evident in later *A. fumigatus* Af293 infection. This study also explored the function of MAIT cells in *A. fumigatus* intratracheal infection and found a possible differences between mice with and without MAIT cells. Although the specific role of MAIT cells in these infection needs further study, findings of this dissertation point out the direction to study their role and propose the possibility of MAIT cells as a potential therapeutic target for clinical fungal infectious diseases.

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