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# ROLE OF MAIT CELLS IN HUMAN ANTIMICROBIAL IMMUNITY

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Front cover: graphical summary of this thesis, including a colored illustration of a MAIT cell and components of the four main studies.
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# Role of MAIT cells in human antimicrobial immunity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By


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*"Para ser grande, sê inteiro: nada*
*Teu exagera ou exclui*
*Sê todo em cada coisa. Põe quanto és*
*No mínimo que fazes.*
*Assim em cada lago a lua toda*
*Brilha, porque alta vive."*

Ricardo Reis
Heteronym of Fernando Pessoa
in "Odes"

*To my Family*

# ABSTRACT

Mucosa-associated invariant T (MAIT) cells are a relatively recently discovered subset of unconventional T cells. In humans, MAIT cells are predominantly $CD8^+CD4^-$ ($CD8^+$) with a smaller $CD8^-CD4^-$ (double-negative, DN) subset, and they are abundant in the peripheral blood, liver, and mucosal tissues. MAIT cells recognize riboflavin metabolites produced by a wide range of bacteria and fungi, and presented by the evolutionarily conserved major histocompatibility complex (MHC) class I-related (MR1) protein. Given the novelty of MAIT cells, this thesis had the overall aim of advancing the knowledge of their immunobiology and antimicrobial immune responses.

In this thesis, we first established experimental platforms to study functions of MAIT cells *in vitro*, including activation, cytokine production, proliferation, cytotoxicity, as well as their ability to kill target cells. The established methodologies are versatile and can be adapted to answer a wide variety of MAIT cell-related questions. We next applied these experimental platforms to study MAIT cell responses to distinct riboflavin biosynthesis-competent microbes, and found them to differ in quality and quantity with the type and dose of microbe. We demonstrated that the TCR β chain composition and the expression of certain natural killer (NK)-cell associated receptors on MAIT cells shape their responses to TCR and innate cytokine stimulation, respectively, and thereby contribute to the functional compartmentalization of this cell population. In the third study, we dissected differences between $CD8^+$ and DN MAIT cells with the aim of understanding the relationship between these subsets. $CD8^+$ MAIT cells display superior functional capacity, consistent with their higher basal levels of co-stimulatory and cytotoxic molecules, and of classical effector transcription factors when compared with DN MAIT cells. Furthermore, DN MAIT cells accumulate during fetal development and their adult Vβ repertoire is a subset of that of $CD8^+$ MAIT cells, suggesting that DN MAIT cells may derive from $CD8^+$ MAIT cells *in vivo*. In the fourth study, we investigated MAIT cells in chronic hepatitis delta virus (HDV) infection. We found that MAIT cells are severely depleted from the peripheral blood of HDV-infected patients in comparison with chronic hepatitis B virus (HBV)-infected patients and healthy controls, and that MAIT cell loss is associated with the severity of liver fibrosis. Residual MAIT cells are activated, exhausted, and functionally impaired in response to TCR stimulation.

Altogether, the findings in this thesis advance our understanding of human MAIT cells as functionally heterogeneous T cells that display differential response patterns to microbes and to innate cytokines, and that are markedly affected in hepatitis delta. At the same time, these findings have given rise to numerous new questions to be addressed in the rapidly expanding field of MAIT cell research in the years to come.

# LIST OF SCIENTIFIC PAPERS

This thesis is based on the publications and manuscripts listed below, which are indicated in the text by Roman numerals.

I. Human MAIT-cell responses to *Escherichia coli*: activation, cytokine production, proliferation, and cytotoxicity
**Joana Dias**, Michał J. Sobkowiak, Johan K. Sandberg, and Edwin Leeansyah
*Journal of Leukocyte Biology* 2016, 100: 233-240

II. Multiple layers of heterogeneity and subset diversity in human MAIT cell responses to distinct microorganisms and to innate cytokines
**Joana Dias**, Edwin Leeansyah, and Johan K. Sandberg
*Proceedings of the National Academy of Sciences of the USA* 2017, 114: E5434-E5443

III. Human CD8-negative MAIT cells are functionally distinct from CD8-positive MAIT cells
**Joana Dias**, Jean-Baptiste Gorin*, Caroline Boulouis*, Robin H. G. A. van den Biggelaar*, Anna Gibbs, Liyen Loh, Douglas F. Nixon, Kristina Broliden, Annelie Tjernlund, Johan K. Sandberg†, and Edwin Leeansyah†
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*Manuscript*

IV. Chronic hepatitis delta virus infection drives severe loss and functional exhaustion of MAIT cells
**Joana Dias**, Julia Hengst, Edwin Leeansyah, Sebastian Lunemann, David F. G. Malone, Svenja Hardtke, Tiphaine Parrot, Lena Berglin, Thomas Schirdewahn, Michael P. Manns, Markus Cornberg, Hans-Gustaf Ljunggren, Heiner Wedemeyer*, Johan K. Sandberg*, and Niklas K. Björkstrom*
*shared last authors
*Manuscript*

# LIST OF ADDITIONAL SCIENTIFIC PAPERS

SI. Will loss of your MAITs weaken your HAART?
Johan K. Sandberg, **Joana Dias**, Barbara L. Shacklett, and Edwin Leeansyah
*AIDS* 2013, 27: 2501-2504

SII. Arming of MAIT Cell Cytolytic Antimicrobial Activity Is Induced by IL-7 and Defective in HIV-1 Infection
Edwin Leeansyah, Jenny Svärd, **Joana Dias**, Marcus Buggert, Jessica Nyström, Máire F. Quigley, Markus Moll, Anders Sönnerborg, Piotr Nowak, and Johan K. Sandberg
*PLoS Pathogens* 2015, 11: e1005072

SIII. Extensive Phenotypic Analysis, Transcription Factor Profiling, and Effector Cytokine Production of Human MAIT Cells by Flow Cytometry
**Joana Dias**, Johan K. Sandberg, and Edwin Leeansyah
*Methods in Molecular Biology* 2017, 1514: 241-256

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Ac-6-FP | Acetyl-6-formyl pterin |
| APC | Antigen-presenting cell |
| ART | Antiretroviral therapy |
| Bak | Bcl-2 antagonist/killer |
| Bax | Bcl-2 associated X, apoptosis regulator |
| BCG | Bacillus Calmette-Guérin |
| *C. albicans* | *Candida albicans* |
| Cas9 | Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 |
| CCR | CC chemokine receptor |
| CD | Cluster of differentiation |
| $CD4^+$ cell | $CD8^-CD4^+$ cell |
| CD40L | CD40 ligand |
| $CD8^+$ cell | $CD8^+CD4^-$ cell |
| CF | Cystic fibrosis |
| cfu | Colony-forming unit |
| CMV | Cytomegalovirus |
| COPD | Chronic obstructive pulmonary disease |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CTV | Cell trace violet |
| CVID | Common variable immunodeficiency |
| CXCR | CXC chemokine receptor |
| DC | Dendritic cell |
| DCF | Diclofenac |
| DCM | Dead cell marker |
| DIG fraction | Detergent insoluble glycolipid-enriched fraction<br>*Also known as raft* |
| DN cell | Double-negative cell (*meaning $CD8^-CD4^-$ cells*) |
| DP cell | Double-positive cell (*meaning $CD8^+CD4^+$ cells*) |
| *E. coli* | *Escherichia coli* |
| *E. faecalis* | *Enterococcus faecalis* |
| Eomes | Eomesodermin |
| ER | Endoplasmic reticulum |
| FACS | Fluorescence-activated cell sorting |

| | |
|---|---|
| FATAL assay | Fluorometric assessment of T lymphocyte antigen specific lysis assay |
| FLICA | Fluorochrome-labelled inhibitor of caspases |
| GEM cell | Germline-encoded mycolyl-reactive cell |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| Gnly | Granulysin |
| Grz | Granzyme |
| *H. pylori* | *Helicobacter pylori* |
| HBsAg | Hepatitis B virus (HBV) surface antigens |
| HBV | Hepatitis B virus |
| HCV | Hepatitis C virus |
| HDAg | Hepatitis delta antigen |
| HDV | Hepatitis delta virus |
| HIV-1 | Human immunodeficiency virus type 1 |
| HMBPP | 4-hydroxy-3-methyl-but-2-enyl pyrophosphate |
| HTLV-1 | Human T-lymphotropic virus type 1 |
| IFN | Interferon |
| IKZF2 | IKAROS family zinc finger 2<br>*Also known as Helios* |
| IL | Interleukin |
| iNKT cell | Invariant natural killer T (NKT) cell |
| KO | Knock-out |
| LDH | Lactate dehydrogenase |
| LPS | Lipopolysaccharide |
| *M. tuberculosis* | *Mycobacterium tuberculosis* |
| MACS | Magnetic-activated cell sorting |
| MAIT cell | Mucosa-associated invariant T cell |
| MDR1 | Multidrug resistance protein 1<br>*Also known as ABCB1 (ATP-binding cassette sub-family B member 1)* |
| MHC | Major histocompatibility complex |
| MHC-Ia | Classical major histocompatibility complex (MHC) class I |
| MHC-Ib | Non-classical major histocompatibility complex (MHC) class I |
| MICA | Major histocompatibility complex (MHC) class I polypeptide-related sequence A |
| MLN | Mesenteric lymph node |
| MR1 | Major histocompatibility complex (MHC) class I-related |
| NF-kB | Nuclear factor-kB |
| NK cell | Natural killer cell |

| | |
|---|---|
| NKT cell | Natural killer T cell |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cell |
| PD-1 | Programmed death-1 receptor |
| PLZF | Promyelocytic leukemia zinc finger |
| | *Also known as ZBTB16 (zinc finger and BTB domain containing 16)* |
| PMA | Phorbol myristate acetate |
| Prf | Perforin |
| RL-6-Me-7-OH | 7-hydroxy-6-methyl-8-D-ribityllumazine |
| RL-6,7-diMe | 6,7-dimethyl-8-D-ribityllumazine |
| RORγt | Retinoid-related orphan receptor γt |
| rRL-6-$CH_2OH$ | Reduced 6-hydroxymethyl-8-D-ribityllumazine |
| *S. aureus* | *Staphylococcus aureus* |
| SEB | Staphylococcal enterotoxin B |
| SNE | Stochastic neighbor embedding |
| TAP | Transporter associated with antigen processing |
| TBX21 | T box transcription factor 21 |
| | *Also known as T-bet* |
| TCR | T cell receptor |
| Th | T helper |
| TIM-3 | T-cell immunoglobulin and mucin domain-containing protein-3 |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| Treg | T regulatory |
| ULBP | UL16-binding protein |
| XIAP | X-linked inhibitor of apoptosis |
| α-GalCer | α-galactosylceramide |
| β2m | β2-microglobulin |
| 293T-hMR1 cell | 293T cells stably transfected with human MR1 (major histocompatibility complex (MHC) class I-related protein) |
| 5-A-RU | 5-amino-6-D-ribitylaminouracil |
| 5-OE-RU | 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil |
| 5-OH-DCF | 5-hydroxy diclofenac |
| 5-OP-RU | 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil |
| 6-FP | 6-formyl pterin |

# 1 INTRODUCTION

In a world abundantly populated by pathogens, the human body has developed multifaceted protective mechanisms that together constitute the *immune system*. This system counts on two main arms to ultimately fight infections. The *innate* immune system comprises anatomical, physiological, and inflammatory barriers, and uses innate immune cells to recognize structural motifs shared by many pathogens – the pathogen-associated molecular patterns (PAMPs) [1]. In this way, rapid responses against a broad range of pathogens can be mounted in an unspecific manner [1]. In contrast, the *adaptive* immune system relies on specialized cells that recognize unique pathogenic motifs, the *antigens* [1]. Such cells with a certain antigen specificity are relatively rare and need to expand upon antigen encounter in order to mount efficient responses [1]. Therefore, adaptive immune responses, although highly specific, take longer to develop [1].

Classical (or conventional) T cells play a pivotal role in adaptive immunity. They express surface αβ T cell receptors (TCRs) that recognize peptide antigens in complex with molecules displayed on the surface of antigen-presenting cells (APCs) [2, 3]. The human TCR repertoire is generated by directed somatic recombination and is highly diverse. In addition, the antigen-presenting molecules are encoded by genes of the major histocompatibility complex (MHC) that are highly polymorphic [2, 3]. This ensures that both the TCRs and the antigen-presenting molecules can bind to virtually any pathogen-derived peptide [2, 3].

In between the fast and broad innate immune system and the slower but highly specific adaptive immune system, there is a group of T cells with both innate and adaptive characteristics. These cells are usually called *unconventional* or *innate-like T cells* [2, 3].

## 1.1 UNCONVENTIONAL T CELLS

Unconventional T cells recognize antigens presented by antigen-presenting molecules encoded by genes that display a low degree of polymorphism [2, 3]. They are abundant in peripheral blood and/or tissues, and are able to quickly respond to antigenic challenges [2, 3]. Several types of human unconventional T cells have been described, including CD1-restricted T cells, HLA-E-restricted T cells, mucosa-associated invariant T (MAIT) cells (all of which are αβ T cells), and γδ T cells.

### 1.1.1 Unconventional αβ T cells

Group 1 (CD1a, CD1b, and CD1c) and group 2 (CD1d) molecules of the CD1 family can present a vast array of self and microbial lipid antigens to αβ T cells [2, 4-6]. Within the group 1 CD1-restricted T cells, CD1b-resticted T cells include, among others, the *germline-encoded mycolyl-reactive* (GEM) [7] and the *LDN5-like* [8] *T cells*, which express conserved

TCRs and recognize glucose monomycolate, a mycobacterial glycolipid [7, 8]. CD1a- and CD1c- restricted T cells can also recognize mycobacterial lipid antigens [9-13]. In addition, CD1a-restricted T cells represent the majority of CD1-restricted autoreactive T cells [14, 15], and recognize a wide variety of endogenous antigens [2, 16].

To date, group 2 CD1-restricted T cells, also called *natural killer T* (NKT) *cells*, are the most extensively studied T cells restricted by molecules of the CD1 family [6]. Type I NKT cells, or *invariant NKT* (iNKT) *cells*, express a semi-invariant TCR (Vα24-Jα18 paired with Vβ11 in humans) [17, 18], and CD161 [19], a receptor expressed on *natural killer* (NK) *cells* and subsets of T cells [19-21]. iNKT cells recognize α-galactosylceramide (α-GalCer), among other lipids [2, 22], and represent approximately 0.1% of T cells in the peripheral blood of healthy adults [23]. In contrast, type II NKT cells are non-invariant and lack the conserved TCR Vα24 segment [2, 24]. These cells do not recognize α-GalCer and, to date, extensive investigation has been carried out on murine type II NKT cells that recognize sulfatide [2, 25].

Besides CD1-restricted T cells, there are also unconventional T cells restricted by the HLA-E molecule, which has been reported to bind peptides derived from MHC class I leader peptides, as well as from cytomegalovirus (CMV) and bacterial pathogens [2, 26-28].

This thesis focuses on human MAIT cells, which are described in detail in *Section 1.2*.

### 1.1.2 Unconventional γδ T cells

γδ T cells, duly named because of their surface expression of γδ as opposed to αβ TCRs, can be found in peripheral blood and tissues [2]. The most abundant γδ T cell population in human peripheral blood expresses a conserved γδ rearrangement and recognizes small, phosphorylated metabolites, which are generally called *phosphoantigens* and are produced by mammalian cells or microbes (*e.g.* 4-hydroxy-3-methyl-but-2-enyl pyrophosphate, or HMBPP, derived from bacteria) [2, 29]. Other γδ T cells recognize CD1a- and CD1d- lipid complexes [30-33], as well as stress-induced proteins, such as MHC class I polypeptide-related sequence A (MICA) and UL16-binding protein (ULBP) [34, 35]. The panel of antigens and antigen-presenting molecules recognized by γδ T cells is very diverse and has been previously reviewed in detail [36, 37].

## 1.2 MAIT CELLS

### 1.2.1 Towards the discovery of MAIT cells

The semi-invariant TCR rearrangement characteristic of MAIT cells in humans, Vα7.2-Jα33, was identified for the first time in 1993 when Porcelli *et al.* [17] examined the TCRα chains of peripheral blood $CD8^-CD4^-$ (double-negative, DN) T cells from healthy individuals [17]. Later in 1999, Tilloy *et al.* [38] reported that this TCR rearrangement defines a new cell population of DN and CD8αα T cells in humans with an effector memory phenotype

(CD45RA$^{lo}$CD45RO$^{+}$) and preferential usage of the TCR Vβ2 or Vβ13 segments [38]. The homologous Vα19-Jα33 rearrangement was found in mice and cattle [38]. Already at this time, it was suggested that these cells were restricted by a distinct non-classical and β2-microglobulin (β2m)-dependent antigen presenting molecule due to their absence in mice lacking β2m and their presence in humans and/or mice lacking MHC class I, MHC class II, CD1, and transporter associated with antigen processing (TAP) molecules [38]. In 2003, Treiner *et al.* [39] identified the MHC class I-related (MR1) protein as the restricting molecule of this cell population due to the absence of Vα19-Jα33 transcripts in MR1-deficient mice [39]. The finding that T cells expressing the Vα7.2-Jα33 and Vα19-Jα33 rearrangements were abundant in human gut biopsies and murine lamina propria, respectively, led these cells to be called *mucosa-associated invariant T* (MAIT) *cells* [39].

### 1.2.2 Evolutionary conservation of MR1 and MAIT cells

The *MR1* gene, discovered in 1995 [40], is believed to have been established 160 to 220 million years ago in a common ancestor of placental and marsupial mammals [41]. MR1 and MAIT cells are present and highly conserved across mammals, and are found not just in humans and mice but also in non-human primates [42-44], cattle, sheep, bats, elephants, Tasmanian devils, and opossums [45, 46]. The degree of interspecies evolutionary conservation is high, as exemplified by the MR1 molecules from humans and mice, which are 90% and 89% identical in the amino acid sequences of their α1 and α2 domains, respectively [47]. Moreover, murine and human MAIT cells are highly cross-reactive to ortholog MR1 molecules [48, 49]. This suggests highly evolutionary conservation of the MR1 antigen presentation to MAIT cells process, and a fundamental role of the MAIT cell-MR1 axis in the immune system.

### 1.2.3 Identification of MAIT cells

In 2009, Martin *et al.* [50] developed the monoclonal antibody (clone 3C10) that recognizes the human TCR Vα7.2 segment and showed that the Vα7.2-Jα33 gene rearrangement characteristic of MAIT cells was only found in Vα7.2$^{+}$ cells expressing high levels of CD161 [50]. Later in 2011, Le Bourhis *et al.* [49] reported that CD161 and interleukin (IL)-18 receptor α (IL-18Rα) were co-expressed on MAIT cells [49]. Thus, co-expression of Vα7.2, and IL-18Rα or high levels of CD161 within the T cell compartment was adopted in the field to identify MAIT cells by flow cytometry. The identification of MAIT cell agonists [51, 52] (described in *Section 1.2.6.*3) led to the generation of fluorescent MR1 tetramers refolded with such compounds [51, 53]. As these reagents have become widely available to the research community, they are being adopted as a preferred tool for the identification of MAIT cells. In healthy adult individuals, the cell population identified using MR1 tetramers greatly overlaps with that defined as CD161$^{hi}$Vα7.2$^{+}$ [51, 53, 54].

As MAIT cells are rare in common laboratory strains of mice [38], most studies on murine MAIT cells have relied on mouse models overexpressing MAIT cells (iVα19 and Vβ6 transgenic mice, iVα19-Vβ6 double-transgenic mice, and more recently CAST/EiJ congenic

mice) [50, 55, 56]. The study of murine MAIT cells has been hampered by the lack of a Vα19-specific antibody, but has recently advanced through the development of murine MR1 tetramers [53, 57].

### 1.2.4 MAIT cell development and phenotype

Like NKT cells, MAIT cells develop in the thymus [38, 50], where they are selected by MR1-expressing $CD8^+CD4^+$ (double-positive, DP) thymocytes [58]. Two studies [59, 60] initially alluded to this through the detection of high levels of endogenous MR1 in mouse and human DP thymocytes [59, 60]. With the use of transgenic mouse models and thymic organ cultures, Seach *et al.* [58] went on to demonstrate an indispensable and non-redundant role of MR1-expressing DP thymocytes in MAIT cell selection in the thymus, whereas thymic B cells, dendritic cells (DCs), and macrophages were not essential for this process [58].

In the thymus and cord blood, human MAIT cells display a $CD45RA^+CD45RO^-$ naïve phenotype [50, 61, 62], whereas adult peripheral blood MAIT cells are $CD45RA^-CD45RO^+CD28^+CCR7^-CD62L^-$ effector memory cells [38, 50, 61] (Figure 1). Cord and peripheral blood MAIT cells are predominantly $CD8^+CD4^-$ ($CD8^+$) and DN with minor $CD8^-CD4^+$ ($CD4^+$) cells. The thymus, however, contains $CD4^+$, $CD8^+$, and DP MAIT cells [50, 63]. Koay *et al.* [63] proposed a three-stage pathway for MAIT cell development in the thymus consisting of $CD161^-CD27^-$ cells (stage 1, predominantly DP and $CD4^+$ in the thymus), $CD161^-CD27^+$ (stage 2, mostly DP, $CD4^+$ and $CD8^+$ in the thymus), and $CD161^+CD27^{pos\text{-}lo}$ cells (stage 3, predominantly $CD8^+$ and DN in the thymus) [63]. While stage 1 MAIT cells are exclusively present in the thymus, stage 2 MAIT cells are also detected in cord and peripheral blood samples from young children, albeit at lower levels, but they are absent in adult peripheral blood [63]. Stage 3 MAIT cells are rare in the thymus and predominate in cord and peripheral blood [63] (Figure 1). Importantly, and in contrast to cord and peripheral blood, MAIT cells in the thymus predominantly lack the expression of CD161 and IL-18R [61, 63], and are only functional at stage 3 [63]. Stage 3 thymic MAIT cells respond to mitogen stimulation at lower levels than their extra-thymic counterparts, which suggests that the process of MAIT cell functional maturation occurs extra-thymically [63].

MAIT cells in the thymus and cord blood exclusively express the CD8αβ co-receptor [62, 63], whereas those in adult peripheral blood can express either CD8αβ or CD8αα [50, 62, 63] (Figure 1). Remarkably, CD8αα expression is mostly restricted to the MAIT cell population [62, 64]. $CD161^{hi}CD8\alpha\beta^+$ and $CD161^{hi}CD8\alpha\alpha^+$ T cells share similar phenotypic and functional characteristics, and $CD161^{hi}CD8\alpha\alpha^+$ T cells could be derived *in vitro* from $CD161^{hi}CD8\alpha\beta^+$ T cells [62]. This observation, together with the exclusive expression of CD8αβ among $CD8^+$ MAIT cells in the cord blood and thymus [62, 63], suggests that $CD8\alpha\alpha^+$ MAIT cells may derive from $CD8\alpha\beta^+$ MAIT cells *in vivo*.

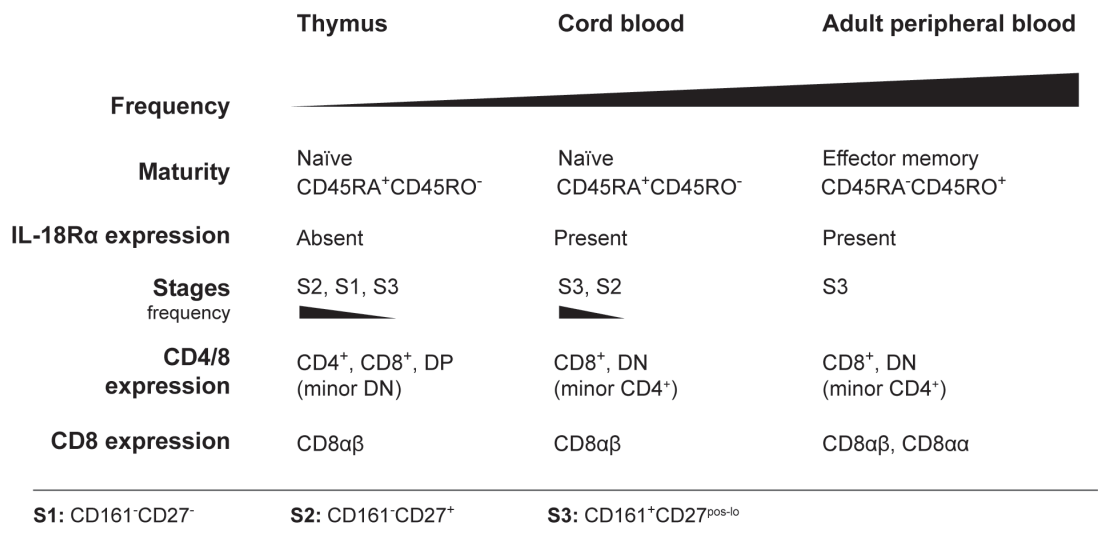


**Figure 1. Phenotype and frequency of human MAIT cells in the thymus, cord blood, and adult peripheral blood.** Expression levels of CD27 in S3 are abbreviated as *pos* (positive) and *lo* (low). *S*, stage.

Thymic MAIT cells express the transcription factors promyelocytic leukemia zinc finger (PLZF or zinc finger and BTB domain containing 16, ZBTB16), retinoid-related orphan receptor (ROR) γt, and T box transcription factor 21 (TBX21 or T-bet) at gradually increasing levels from stage 1 to stage 3 [63]. In adult peripheral blood, MAIT cells express PLZF, likely responsible for their effector memory phenotype, and RORγt [50, 61, 65, 66, paper SII], a T helper (Th) 17 cell-associated transcription factor [67]. Adult circulating MAIT cells also express T-bet, Helios (or IKAROS family zinc finger 2, IKZF2), and eomesodermin (Eomes) at low, intermediate and high levels, respectively [65, paper SII]. Eomes and T-bet are reciprocally expressed by memory and effector $CD8^+$ T cells [68], whereas Helios expression has been associated with T cell activation and proliferation [69].

In addition to the IL-18R, adult peripheral blood MAIT cells also express receptors for IL-12, IL-23, and IL-7 [61, 70, 71, paper SII]. Furthermore, they express the NK cell receptor NKG2D and CD26 [61], which is a dipeptidase and co-stimulatory molecule [72]. Interestingly, MAIT cells express high levels of the multidrug resistance protein 1 (MDR1, also known as ATP-binding cassette sub-family B member 1 (ABCB1)) [61], which is a multidrug efflux protein [73, 74]. Figure 2 summarizes the MAIT cell phenotype in the peripheral blood of healthy adult individuals.

MAIT cells have also been studied in second-trimester fetal tissues, including the thymus, secondary lymphoid organs (spleen and mesenteric lymph nodes, MLNs), and peripheral organs (liver, lung, and small intestine) [75]. Fetal $CD8\alpha\alpha^+$ and $IL\text{-}18R\alpha^+$ MAIT cells, which display a more mature (effector memory) $CD45RO^+CD62L^-PLZF^{hi}$ phenotype than their negative counterparts, are preferentially enriched in the peripheral organs [75]. MAIT cells in the peripheral fetal organs are also more functional following bacterial stimulation than those in the secondary lymphoid organs and in the thymus [75]. This is consistent with the notion that functional maturation occurs outside the thymus [63], and suggests that MAIT cell maturation occurs in the fetus prior to bacteria exposure and establishment of the commensal

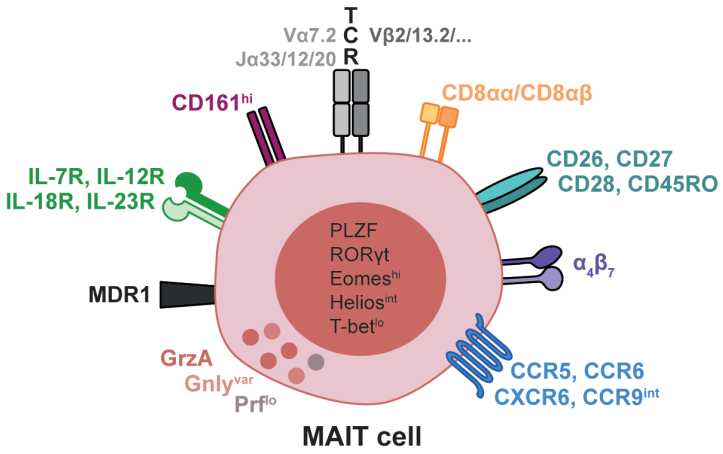


**Figure 2. Phenotype of peripheral blood MAIT cells in healthy adult individuals.** Expression levels are abbreviated as *hi* (high), *int* (intermediate), *lo* (low), and *var* (variable).

microflora. Considering that cord blood MAIT cells are naïve, this may suggest the existence of two distinct MAIT cell subsets in adult individuals: a tissue-resident and non-recirculating effector memory MAIT cell subset established before birth, and a naïve MAIT cell subset that will mature after birth [76].

In the thymus and cord blood, MAIT cells are found at relatively low levels [50, 63] (Figure 1). While thymic MAIT cell levels remain low and stable over time [63], the peripheral blood MAIT cell population gradually expands until 20-40 years of age, after which it contracts [77-79]. Peripheral blood MAIT cells reach frequencies approximately ten times higher than those in the thymus and cord blood [63], ranging between 1 and 10% of the total circulating T cells [50, 61] with high inter-individual variability [77, 78]. The levels of $CD8^+$ and $CD4^+$ MAIT cells inversely decrease and increase with age, respectively, and women of reproductive age (15 to 50 years old) were reported to have significantly more MAIT cells than men [77, 78]. Notably, while MAIT cells represent only a minor fraction (≈ 10%) of the $CD161^{hi}CD8\alpha^+$ T cell pool in cord blood, they cover the vast majority of these T cells (≈ 90%) in adult individuals [62].

### 1.2.5 MAIT cell tissue localization

Adult peripheral blood MAIT cells express a distinct combination of chemokine receptors (Figure 2) that mediate their trafficking to peripheral tissues (Figure 3). This includes the expression of CCR6 and CXCR6 [61], liver-homing chemokine receptors [80-82], as well as α4β7 [39] and intermediate levels of CCR9 [61], receptors involved in lymphocyte migration to the gut [83-85]. Indeed, MAIT cells are highly enriched in the liver, where they constitute 15% to 50% of hepatic T cells [61, 70, 86-89] and represent the predominant T cell population expressing CD161 and CD56 [89]. They are also present at variable frequencies within the gut. In the small intestine, MAIT cells have been found in the duodenum (≈ 2% of T cells) [90], jejunum (≈ 60% of $CD4^-$ T cells) [53], and ileum (≈ 1.5% of T cells) [91], whereas in the large intestine they are present in the colon (≈ 10% of T cells) [87, 92], and in the rectum (≈ 2% of T cells) [93]. The expression of CXCR6 and CCR5 [61] also indicates their ability to traffic to the lungs [94] (≈ 2% of T cells in sputum and bronchoalveolar

lavage, and ≈ 4% of T cells in endobronchial biopsies) [95, 96] (Figure 3). MAIT cells have also been detected in the stomach (≈ 2.5% of T cells) [97], the endometrium and cervix (≈ 1 to 2% of T cells) [65], and the skin [98, 99]. Transcripts for the MAIT cell TCR were also detected in the kidneys, ovaries, and prostate [100]. In contrast, MAIT cells are rarely found in lymph nodes [61] due to their lack of CD62L and CCR7 expression [61, 101-104].

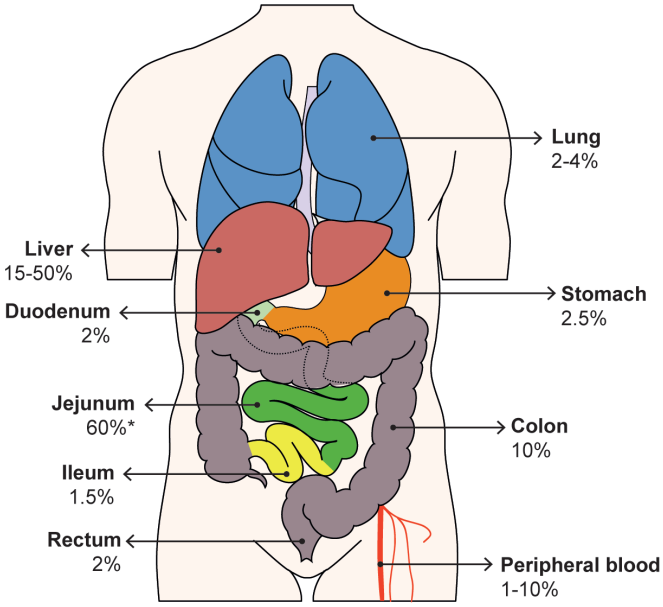


**Figure 3. Tissue distribution of MAIT cells in healthy adult individuals.** Approximate frequencies of MAIT cells within total $CD3^+$ cells are indicated, except for MAIT cells in the jejunum where the frequency (*) has been determined within $CD3^+CD4^-$ cells.

## 1.2.6 Antigen presentation to MAIT cells

### *1.2.6.1 MR1*

*MR1* is a non-polymorphic gene located on chromosome 1 in humans [40], similar to the *CD1* gene [105], and outside of the MHC located on chromosome 6 [106]. Surprisingly, however, the MR1 molecule shares higher homology in its α domains with classical MHC class I (MHC-Ia) molecules compared with other non-classical MHC class I (MHC-Ib) molecules [40].

Four human MR1 isoforms have been identified that are generated through alternative splicing and are denoted MR1A to MR1D [47]. MR1A corresponds to the full-length protein that was originally discovered, and is made up of 341 amino acids and all of the structural domains of a MHC-Ia molecule: namely a signal peptide, three extracellular domains (α1 and α2, which form the ligand-binding pocket, and α3, which interacts with β2m), a transmembrane domain, and a cytoplasmic domain [40, 47, 52, 107, 108]. The other three isoforms lack the α3 domain [47], and the MR1C transcript also lacks the transmembrane domain, thereby potentially encoding a soluble protein [47]. Similar to MR1A, MR1B is a functional antigen-presenting molecule capable of MAIT cell activation that is expressed on the cell surface as a homodimer and in the absence of β2m [47, 109, 110].

Although human MR1 transcripts are ubiquitously expressed [40, 47, 110], basal surface expression of MR1 (*i.e.*, at steady state) has been difficult to detect on non-MR1 transfected cells [46, 59, 110-113], and reports on its intracellular location are controversial. Some studies suggest that MR1 is predominantly retained in a pre-Golgi compartment, namely the endoplasmic reticulum (ER) [108, 114], in a ligand-receptive and incompletely folded state with no β2m association [114]. In contrast, others have reported that MR1 is located both in the ER, and in late endosomes and lysosomes where it associates with β2m [115, 116]. MR1 predominantly binds to soluble ligands (described in *Section 1.2.6.3*) in the ER via the formation of a Schiff base (covalent bond) between the positively charged amino group of lysine-43 (K43) and the ligand, which in turn neutralizes the positive charge in K43 [114]. This promotes complete folding of MR1, association of MR1 with β2m [114], and egress of the MR1-β2m-ligand complex, which leads to its rapid upregulation on the cell surface [114, 115]. The ternary complex is then internalized and degraded in late endosomes and lysosomes [114], with only a small fraction of the internalized MR1 reportedly exchanging ligands and recycling back to the cell surface [114]. In addition to the ER, Harriff *et al.* [115] reported that endocytic compartments can also function as a source of MR1 molecules available to bind soluble ligands before translocation to the cell surface [115]. Differences between the aforementioned studies both at steady state and in the presence of soluble ligands may be due to the presence [108, 115, 116] or absence [114] of soluble MR1 ligands in the culture medium, the effect of MR1 overexpression [108, 114, 116] and of molecular tags in MR1 trafficking [115, 116], and the cell types used in the assays [117].

A few MR1 molecules can leave the ER and bind to soluble ligands directly on the cell surface [114]. This is in agreement with the capacity that fixed APCs have to activate MAIT cells in the presence of ligand-producing microbes [49], and may represent an important pathway for presentation of ligands that may not be able to reach the ER or endosomes in sufficient concentrations to bind to MR1 [3]. Nonetheless, the contribution of surface loading to the overall ligand presentation by MR1 is probably limited in most circumstances [114].

Microbe-associated ligands (*i.e.*, ligands from intracellular or phagocytosed microbes) appear to be loaded and presented by MR1 via endosomes through a different pathway than that utilized for soluble ligands [113, 115]. In support of this notion, the trafficking molecules involved in these processes were reported to be different [115], and inhibitors of phagocytosis and endolysosomal acidification decreased MAIT cell activation in response to ligand-producing microbes but not to microbial supernatants [49, 113].

MR1 surface expression increases upon ligand availability, and its surface expression was shown to depend on nuclear factor-kB (NF-kB)-mediated activation of APCs [113]. In addition, toll-like receptor (TLR)-mediated stimulation of APCs can also increase MR1 surface expression [113, 118].

#### *1.2.6.2 MAIT cell TCR*

Most MAIT cells express the TCRα chain defined by the Vα7.2-Jα33 rearrangement [38, 53], whereas a minority expresses either Vα7.2-Jα12 or Vα7.2-Jα20 instead [53, 100]. Overall, the Jα12 and Jα20 sequences are very similar to Jα33, and, importantly, they retain the tyrosine-95 (Y95) residue within the CD3Rα loop, which is crucial for MAIT cell activation (as described in *Section 1.2.6.4*) [53]. These TCRα chains predominantly pair with Vβ2 or Vβ13.2, although additional Vβ diversity has been described for MAIT cells with the Vα7.2-Jα33 rearrangement [38, 53, 100, 119].

#### *1.2.6.3 MR1 ligands & MAIT cell agonists and antagonists*

In 2012, a seminal paper by Kjer-Nielsen *et al.* [52] described the first MR1 ligands with the capacity to activate MAIT cells. These were compounds derived from the riboflavin (or vitamin $B_2$) biosynthesis pathway known as ribityl lumazines: reduced 6-hydroxymethyl-8-D-ribityllumazine (rRL-6-$CH_2$OH), 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH), and 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe) [52]. Subsequently, the pyrimidines 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) were identified as highly potent MAIT cell agonists [51]. These pyrimidines are formed by a non-enzymatic reaction between 5-amino-6-D-ribitylaminouracil (5-A-RU), a riboflavin precursor, and either methylglyoxal or glyoxal [51], two ubiquitous molecules produced during several metabolic pathways in microbes or humans, including glycolysis [3, 120]. Both 5-OE-RU and 5-OP-RU are very unstable, especially in acidic aqueous medium, and they quickly undergo dehydration to form the stable ribityl lumazines [51]. However, they can be captured and stabilized by MR1, and then function as potent MAIT cell activating antigens [51].

Riboflavin is produced by plants, as well as by bacteria and fungi [121, 122]. Therefore, MAIT cells can be activated in an MR1-dependent manner by microorganisms that possess the riboflavin biosynthesis pathway, including *Escherichia*, *Pseudomonas*, *Klebsiella*, *Lactobacillus*, *Staphylococcus*, *Mycobacteria*, and *Salmonella* species of bacteria, and *Candida* and *Saccharomyces* species of fungi, but not by microbes that lack the ability to produce riboflavin, such as *Enterococcus faecalis*, *Streptococcus* group A, and *Listeria monocytogenes* [49, 52, 123]. Interestingly, because MR1 ligands are secreted and diffusible, it has been hypothesized that MAIT cells may sense microbial infections across mucosal membranes [52]. As mammals are unable to produce riboflavin, MAIT cell recognition of microbial riboflavin derivatives provides another basis for immune-mediated self *vs.* non-self discrimination [124].

A group of MR1 ligands with the capacity to upregulate MR1 surface expression without activating MAIT cells has been described. These compounds are MAIT cell antagonists, which inhibit MAIT cell activation by competing with MAIT cell agonists for the MR1 binding pocket [3], and they derive from folic acid [52, 125, 126], a constituent of the diet and of culture media such as RPMI-1640 [3, 52]. The first MAIT cell antagonist identified

was 6-formyl pterin (6-FP) [52], which is spontaneously generated by the photodegradation of folic acid [127]. Later on, acetyl-6-FP (Ac-6-FP), an acetylated analog of 6-FP, and two other variants of 6-FP, 2-acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal and 2-acetylamino-4-hydroxy-6-formylpteridine, were described as more potent MAIT cell antagonists that upregulate MR1 to a greater extent than 6-FP [126].

The identification of MR1 ligands has made possible the generation of MR1 tetramers loaded with rRL-6-$CH_2OH$ [53], 5-OE-RU, and 5-OP-RU [51], which efficiently stain all human MAIT cells, contrarily to the MR1 tetramers loaded with 6-FP and Ac-FP [125]. MR1 tetramers loaded with 5-OP-RU (and with 6-FP for use as a negative control) are presently used to specifically detect and study MAIT cells. Furthermore, an MR1 ligand analogue of 5-OP-RU that displays greater stability in water has been synthesized, and it is capable of MR1 surface upregulation and MAIT cell activation [128]. More recently, a new panel of MR1 ligands has been identified that includes drugs, drug metabolites, and drug-like molecules, such as diclofenac (DCF) and salicylates, with differential capacities to upregulate MR1 and activate or inhibit MAIT cells [129]. Overall, the MR1 ligands identified to date suggest that MR1 can accommodate a heterogeneous panel of compounds, which opens a window of possibilities for the modulation of MAIT cell activity *in vitro* and *in vivo* [129]. To date, no endogenous MR1 ligands have been identified.

#### *1.2.6.4 MAIT cell TCR recognition of MR1-ligand complexes*

The MAIT cell agonists 5-OE-RU and 5-OP-RU and the antagonists 6-FP and Ac-6-FP covalently bind to MR1 via the formation of a Schiff base with the K43 residue of MR1, which demonstrates a strong association between MR1 and the ligand [51, 52, 125]. Schiff base formation triggers the molecular alterations necessary for MR1 to traffic to the cell surface (as described in *Section 1.2.6.1*) [114], and is, therefore, essential for efficient MR1 surface expression [3]. In agreement with this, the ribityl lumazine RL-6-Me-7-OH, DCF and its metabolite 5-hydroxy DCF (5-OH-DCF), which establish multiple contacts with MR1 without the formation of a Schiff base [3, 51, 129], are less potent inducers of MR1 surface upregulation than 5-OP-RU [129].

The MAIT cell activating ribityl lumazines and pyrimidines structurally resemble 6-FP but contain an extra ribityl moiety that allows direct contact with the MAIT cell TCR [51, 52, 130]. This occurs through formation of a direct hydrogen bond between the ribityl moiety and the Y95 residue located in the CDR3α loop of the MAIT cell TCR, and was uniformly demonstrated for the activating antigens rRL-6-$CH_2OH$, RL-6-Me-7-OH, 5-OE-RU, and 5-OP-RU [51, 130, 131]. Interestingly, the importance of Y95 was first hinted before the identification of any MR1 ligands, when Reantragoon *et al.* [132] studied the recognition of human MR1 by the MAIT cell TCR through site-directed mutagenesis of several residues in the TCRα and β chains [132]. In contrast, recognition of DCF and 5-OH-DCF does not involve the formation of a hydrogen bond with the Y95 residue of the TCR [129].

### 1.2.7 MAIT cell effector functions

#### *1.2.7.1 Upregulation of activation markers and production of cytokines*

MAIT cells respond to riboflavin-producing microbes through the upregulation of the activation markers CD69 and CD25 (or IL-2Rα chain) [49, 92, 93, 133] and the secretion of cytokines. Peripheral blood MAIT cells produce high levels of the Th1 cytokines interferon (IFN) γ and tumor necrosis factor (TNF) [49, 53, 61, 70, 75, 92, 93, 123, 134] (Figure 4), but little or no Th17 cytokines, including IL-17A and IL-22 [61, 75, 92, 93], despite the constitutive expression of the transcription factor RORγt [61, 65, paper SII]. They can, however, produce IL-17A following stimulation with phorbol myristate acetate (PMA)/ionomycin [61, 89], albeit at lower levels than liver MAIT cells [89], which represent the main IL-17-producing T cell population in that organ [89]. Liver MAIT cells also produce IFNγ following microbial stimulation [70, 89]. In contrast, MAIT cells from the female genital tract (endometrium and cervix) display a distinct Th17 cytokine profile in response to microbes, with higher production of IL-17A and IL-22 and lower production of IFNγ and TNF than peripheral blood MAIT cells [65].

Production of the Th2 cytokines IL-4, IL-5, IL-9, and IL-13, as well as of the T regulatory (Treg) cytokine IL-10 by peripheral blood and liver MAIT cells is low or non-existent [61, 89, 135-137]. However, MAIT cells in adipose tissue were reported to produce high levels of IL-10 following PMA/ionomycin stimulation [135]. Also, IL-2 expression by liver and blood MAIT cells was only detected after stimulation with PMA/ionomycin [53, 61, 89] or with superantigens [138]. The latter are potent exotoxins secreted by bacteria including *Streptococcus pyogenes* and *Staphylococcus aureus* that cross-link TCRs on a significant proportion of T cells and MHC class II molecules on APCs, resulting in massive activation of these cells and release of pro-inflammatory mediators [139, 140].

Activated MAIT cells can also produce granulocyte-macrophage colony-stimulating factor (GM-CSF), which is involved in MAIT cell cross-talk mechanisms with other cell types [141, 142]. The combined production of IFNγ, TNF, and GM-CSF by MAIT cells *in vitro* was shown to mediate survival, activation, and differentiation of neutrophils into APC-like cells capable of both exogenous antigen processing and priming of conventional T cells, ultimately resulting in T cell activation and proliferation [141]. In another study, GM-CSF produced by MAIT cells induced differentiation of monocytes into DCs *in vitro* and in a murine model of pulmonary infection *in vivo*. In this mouse model, DCs were in turn involved in the recruitment of activated $CD4^+$ T cells to the site of infection [142]. In addition, activated human MAIT cells have recently been shown to induce maturation of DCs *in vitro* in an MR1- and CD40 ligand (CD40L)- dependent manner [143]. Altogether, this indicates that MAIT cells can link mechanisms of innate and adaptive immunity, which contributes to their involvement in microbial infections.

### *1.2.7.2 Degranulation and killing*

Following microbial stimulation, MAIT cells not only produce cytokines but also degranulate and kill infected target cells (Figure 4). Resting peripheral blood MAIT cells express granzyme (Grz) A, variable levels of granulysin (Gnly), low levels of perforin (Prf), and virtually no GrzB [133, 144, paper SII] (Figure 2). Prf is a membrane pore-forming protein that ultimately allows release of Grz and Gnly molecules into the cytoplasm [145]. While GrzB potently and rapidly induces cell death by apoptosis [145], the cytotoxic capacity of human GrzA is minimal [145, 146]. Gnly is an antibacterial protein that kills intracellular bacteria by damaging their membranes [145, 147]. At baseline conditions, Prf is co-expressed with GrzA and Gnly [paper SII], and GrzA co-localizes with CD107a [144]. This indicates that MAIT cells contain a readily available pool of cytotoxic molecules that can be rapidly released upon degranulation. Following activation, MAIT cells degranulate as indicated by the increased expression of CD107a [70, 133, 134, 144, paper SII], lose GrzA and Gnly [144, paper SII], and upregulate GrzB and Prf [61, 144, paper SII]. Importantly, the $CD107a^{+}GrzA^{lo}$ MAIT cells concomitantly express GrzB and Prf, which indicates that MAIT cells exocytose these molecules upon stimulation [paper SII] (Figure 4). This in turn associates with their capacity to kill target cells *in vitro*, as demonstrated by the release of the cytoplasmic protein lactate dehydrogenase (LDH) in the supernatant of MAIT cell co-cultures with infected target cells [133], the fluorometric assessment of T lymphocyte antigen specific lysis (FATAL) assay [144, 148], and the flow cytometric evaluation of the levels of dead target cells [paper SII]. Importantly, resting MAIT cells are not efficient killer cells due to their lack of GrzB and lower levels of Prf at baseline conditions, when compared with conventional $CD8^{+}$ T cells [144, paper SII].

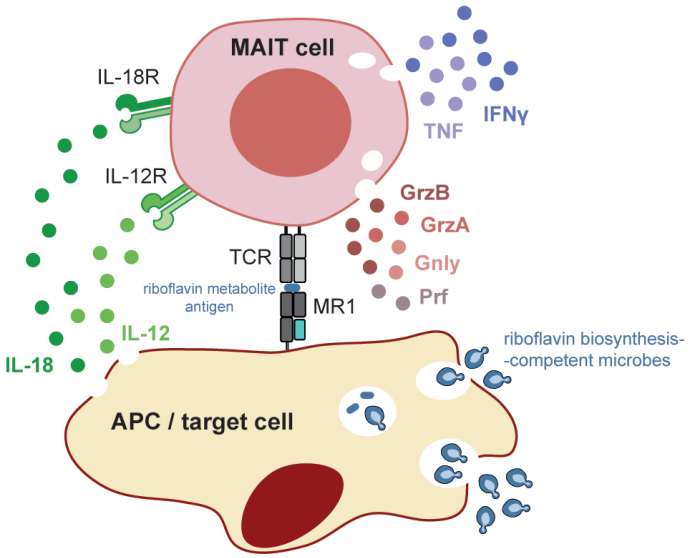


**Figure 4. Summary illustration of the effector functions of peripheral blood MAIT cells following stimulation with riboflavin biosynthesis-competent microbes**. Following microbial stimulation, MAIT cells are able to produce IFNγ and TNF, as well as to degranulate, release GrzA, GrzB, Gnly, and Prf, and kill target cells. MAIT cell responses result from TCR-antigen-MR1 interactions and from the direct effect of APC-derived innate cytokines, such as IL-12 and IL-18, on MAIT cells.

#### *1.2.7.3 Proliferation*

Peripheral blood MAIT cells are also able to proliferate *in vitro* in response to microbial stimulation [75, 144, paper SII], and this occurs despite their lack of basal Ki-67 expression *ex vivo* [61, 144]. MAIT cells upregulate Ki-67 after microbial stimulation, and cells that have proliferated retain their cytolytic profile with high levels of GrzB and Prf [144].

As MR1 is ubiquitously expressed [40, 47, 110], the ability of many different cell types to function as APCs in studies of human MAIT cell responses to microbial stimulation has been demonstrated. These include monocytes [49, 61, 138, 149], macrophages [70, 149], DCs [123, 149, 150], B cells from the blood [134] and liver [70], and epithelial cells from the bile ducts [70] and lungs [123, 150].

#### *1.2.7.4 MR1-dependency of MAIT cell responses to microbes*

The MAIT cell effector functions in response to microbial stimulation described above can result from TCR-antigen-MR1 interactions (*MR1-dependent* responses) and from the direct effect of cytokines produced by APCs, such as IL-12 and IL-18, on MAIT cells (*MR1-independent* responses) (Figure 4). Some functions, including the production of IFNγ, TNF, IL-17 [49, 65, 70, 89, 93, 123, 134], degranulation, loss of GrzA, killing, and proliferation [70, 133, 144, paper SII], are more MR1-dependent than others, such as the upregulation of GrzB and Prf, and the production of IL-22 [65, 144, paper SII]. Previous studies [88, 151] have shown that the short-term IFNγ production by peripheral blood and liver MAIT cells in response to riboflavin biosynthesis-competent *Escherichia coli* was predominantly MR1-dependent, whereas the long-term response was both MR1-dependent and –independent [88, 151]. In contrast, the response to riboflavin biosynthesis-incompetent *E. faecalis* was solely MR1-independent, resulting from the action of IL-12 and IL-18 on MAIT cells [88, 151].

#### *1.2.7.5 MR1-independent MAIT cell responses to innate cytokines*

MAIT cells express IL-12R and IL-18R [49, 70, 71], the latter at higher levels than conventional $CD8^+$ T cells, and can produce IFNγ in response to IL-12 and IL-18 in a process independent of MR1 and TCR signaling [151].

Other cytokines can also exert varying effects on MAIT cells. In the absence of microbial stimulation, IL-15 in synergy with IL-18 and/or IL-12 activates peripheral blood MAIT cells to produce IFNγ and GrzB and to upregulate CD69 [149, 152, 153]. Remarkably, MAIT cells constitute the predominant IFNγ-producing T cell population in response to IL-15 stimulation [152]. On the other hand, IL-7 induces production of GrzB and enhances the expression of Prf and the transcription factors PLZF, RORγt, T-bet, Eomes, and Helios without concomitant production of IFNγ, TNF, or IL-17A [paper SII]. Following suboptimal stimulation with *E. coli*, both IL-15 and IL-7 augment the expression of cytokines and cytolytic molecules [152, paper SII], thereby also increasing the killing capacity of MAIT cells [paper SII]. Notably, IL-7 or the combination of IL-1β and IL-23 were also shown to

enhance IFNγ and IL-17 production by liver MAIT cells following anti-CD3/CD28 TCR-mediated stimulation [89].

In a recent study, Shaler *et al.* [138] studied the response of MAIT cells to bacterial superantigens. MAIT cells respond to staphylococcal enterotoxin B (SEB) by producing IFNγ, TNF, and IL-2 in a process independent of MR1, but dependent on HLA class II, IL-12, and IL-18 [138]. Notably, the MAIT cell response to SEB was more potent than that mounted by iNKT, γδ, and conventional T cells [138].

The capacity of MAIT cells to undergo cytokine-mediated activation also allows them to respond to viruses *in vitro* [149, 154]. MAIT cells respond to dengue virus, influenza virus, and hepatitis C virus (HCV) by producing IFNγ and GrzB [149, 154]. IL-12, IL-18, and IL-15 blocking experiments showed that the MAIT cell IFNγ production predominantly depends on IL-12 and IL-18, IL-18 alone, and IL-18 and IL-15, in response to dengue virus, influenza virus, and HCV, respectively [149, 154]. IFNα and IFNβ, both key players in anti-viral immune responses [155], also activate MAIT cells *in vitro* when in combination with IL-12 or IL-18, and further contribute to the MAIT cell responses to HCV [149]. Importantly, activated MAIT cells inhibit HCV replication *in vitro* via IFNγ production [149, 154].

The activation of MAIT cells by TLR agonists, which may occur during microbial or viral stimulations, is also driven by cytokines in an MR1-independent manner [88, 151]. Agonists for TLR3, TLR4 (lipopolysaccharide, LPS) and TLR8 (single-stranded $RNA_{40}$) activate peripheral blood and liver MAIT cells to produce IFNγ via IL-12 and IL-18 [88, 149, 151].

#### *1.2.7.6 Regulation and modulation of MAIT cell effector functions*

The interplay between MAIT cells, APCs, microbes, and cytokines indicates the existence of several levels at which MAIT cell responses can be regulated. Slichter *et al.* [153] demonstrated that cytokines alone, but not TCR stimulation alone, are sufficient to induce MAIT cell production of IFNγ and GrzB, and that both types of stimuli synergize to induce potent MAIT cell responses [153]. This is consistent with the low MAIT cell responses reported following anti-CD3/28 stimulation alone [53, 61, 89], and with the notion that the production of inflammatory mediators is tightly regulated in order to prevent inflammatory responses to commensal riboflavin biosynthesis-competent microorganisms.

The expression of CD161 can modulate MAIT cell responses although its immunomodulatory effects are, thus far, controversial. Upon anti-CD3/28 TCR stimulation, ligation of CD161 decreased the expression of activation markers and cytokines but did not affect the cytolytic ability of MAIT cells in one study [133], whereas, in another [119], it increased the expression of cytokines. More studies are warranted to clarify the modulatory role of CD161 in MAIT cell responses.

### 1.2.8 Atypical MAIT cells and other MR1-restricted T cells

The MAIT cell population was recently extended after the identification of MAIT cells capable of recognizing not only 5-OP-RU but also 6-FP or Ac-6-FP [156]. Moreover, non-classical MR1-restricted T cells (*i.e.*, T cells restricted by MR1 but with TCR rearrangements different from those described for MAIT cells) have been reported to recognize riboflavin or folate metabolites [156], respond to *Streptococcus pyogenes* (a riboflavin biosynthesis-incompetent microbe) in an MR1-dependent manner (which suggests that MR1 can present microbial activating ligands other than riboflavin metabolites) [157], or respond to non-microbial antigens [158]. Altogether, these findings broaden the definition of MR1-restricted T cells to include other cells that may not express the TCR Vα7.2 segment and/or recognize microbial riboflavin metabolites.

### 1.2.9 MAIT cell antimicrobial role *in vivo*

The high evolutionary conservation of the MAIT cell-MR1 axis among mammals and the ability of MAIT cells to recognize intermediates of the riboflavin biosynthesis pathway, which is conserved among many different species of bacteria and fungi, suggest that MAIT cells play an essential role in host protection against microbes [159].

#### *1.2.9.1 Bacterial infections*

Studies using WT and MR1 knock-out (KO, $MR1^{-/-}$) mice indicated that MAIT cells have a protective role in bacterial infections. A higher bacterial load was detected in the spleens of $MR1^{-/-}$ mice after infection with *E. coli* [49] and *Mycobacterium abscessus* [49], as well as in the lungs following infection with *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) [160]. Moreover, $MR1^{-/-}$ mice not only had a higher bacterial burden, but also succumbed to infections by *Francisella tularensis* [161] and *Klebsiella pneumoniae* [162] at a higher rate than WT mice.

Examinations of MAIT cells in patients suffering from diverse bacterial infections have shown this cell population to be markedly affected. Levels of MAIT cells are lower in the peripheral blood but higher in the lungs of patients with active *Mycobacterium tuberculosis* infection [49, 123, 163, 164], which suggests recruitment of these cells to the site of infection. Interestingly, two studies showed that the decline in peripheral blood MAIT cells selectively occurs in patients with active infection, and not in those with latent infection [123, 163]. In cystic fibrosis (CF) patients, the levels of peripheral blood MAIT cells are also lower than in healthy controls [165]. This decline is associated with disease severity and more pronounced in CF patients infected with *Pseudomonas aeruginosa* [165].

In addition to pulmonary infections, several studies have reported the involvement of MAIT cells in gastrointestinal infections. MAIT cell levels are reduced in the peripheral blood of *Helicobacter pylori*-infected volunteers, with no apparent recruitment to the gastric mucosa [97]. They are also decreased in the peripheral blood of *Vibrio cholerae* O1-infected children [166] and in volunteers who were orally challenged with an attenuated strain of *Shigella*

*dysenteriae 1* [133]. Interestingly, in these studies, the presence of activated MAIT cells positively correlated with the development of *V. cholerae*- or *S. dysenteriae*- specific IgA antibodies, respectively [133, 166], suggesting that MAIT cells may be involved in protective antibody-mediated responses against enteric pathogens [159]. Consistent with these findings, Bennett *et al.* [167] have recently shown that supernatants from activated MAIT cells promote plasmablast differentiation, as well as IgA, IgG, and IgM antibody production *in vitro* [167]. The levels of $CD8^+$ MAIT cells are also lower in the peripheral blood of *Salmonella enterica* serovar Typhi-infected volunteers who developed typhoid fever, but not in those who did not develop typhoid disease [168], again suggesting the involvement of MAIT cells in enteric infections.

Analysis of MAIT cells in severely ill patients revealed that the levels of MAIT cells in peripheral blood are dramatically decreased in patients with bacterial infections, and the extent of decline is bigger in those with non-streptococcal infections [169]. Notably, the development of nosocomial infections was more likely to occur in patients with persistent MAIT cell depletion, in contrast to those where MAIT cell levels increased over time [169]. This suggests a protective role of MAIT cells in severe bacterial infections. Another study, however, showed that in peritoneal dialysis, patients who developed acute peritonitis caused by riboflavin biosynthesis-competent microbes have MAIT cell accumulation in the peritoneal cavity, where they produce IFNγ and TNF, and promote local inflammation [170]. More studies are thus warranted to ascertain the precise role of MAIT cells (protective, pathogenic, or modulatory) in the different types of bacterial infections.

#### *1.2.9.2 Fungal infections*

Several species of fungi, including *Candida albicans* and *Saccharomyces cerevisiae*, possess the riboflavin biosynthesis pathway and can activate MAIT cells *in vitro* [49, 52]. However, to date, the role of MAIT cells in fungal infections in either humans or in animal models has not been investigated.

#### *1.2.9.3 Parasitic infections*

So far only one study has investigated MAIT cells in parasitic infections. Mpina *et al.* [171] reported that following intradermal administration of a high dose of *Plasmodium falciparum* sporozoites to Tanzanian volunteers, peripheral blood MAIT cell levels decreased during early blood-stage parasitemia (11 to 18 days post-infection). Surprisingly, after treatment, MAIT cells rebounded and were maintained in levels higher than those initially measured up to several months post-infection [171].

#### *1.2.9.4 Viral infections*

Although viruses do not produce riboflavin metabolites, the MAIT cell compartment is markedly affected in several human viral diseases. MAIT cells were found to be depleted in the peripheral blood of patients infected with human immunodeficiency virus type 1 (HIV-1), as reported in numerous studies [86, 92, 93, 172-174]. These findings were confirmed by

studies at the mRNA and gDNA level of the presence of the Vα7.2-Jα33 rearrangement [174]. The decline in MAIT cell levels is not reverted with effective antiretroviral therapy (ART) [86, 92, 93, 172]. Interestingly, in perinatally HIV-1-infected children, $CD8^+$ MAIT cells are also lost from the periphery, but gradually recover with ART [175]. In contrast to peripheral blood, the levels of MAIT cells in rectal mucosa and colon of HIV-1-infected patients seem relatively well preserved [92, 93], despite the selective loss of $CD4^+$ MAIT cells in the rectal mucosa that is in agreement with the overall loss of rectal mucosal $CD4^+$ T cells during HIV-1 infection [93]. In HIV-1/*M. tuberculosis* co-infection (both active or latent bacterial infections), the levels of $CD161^{++}CD8^+$ T cells in healthy individuals were detected at low levels similar to those detected in HIV-1 mono-infection [176].

MAIT cells have also been studied in infections caused by dengue virus [149], influenza virus [149, 154], and human T-lymphotropic virus type 1 (HTLV-1) [177], a delta retrovirus that has been implicated in several neoplasms, inflammatory syndromes, and opportunistic infections [178]. In these viral infections, the levels of circulating MAIT cells are also decreased when compared with healthy controls [149, 154, 177]. Interestingly, in one study on patients with severe influenza infections, MAIT cells were found at similar levels in both healthy controls and patients that survived, but were markedly decreased in those who succumbed to the infection, suggesting that MAIT cells may play a protective role in human influenza [154].

Furthermore, MAIT cells have also been shown to be involved in hepatitis. MAIT cell levels are decreased in the blood [86, 149, 179-182] and liver [86, 180] of chronic HCV-infected patients. While the decline in circulating MAIT cells in blood appears to be independent of the stage of liver fibrosis [179], their levels in the liver were found to inversely correlate with liver inflammation and fibrosis in one study [180]. Both residual circulating and liver MAIT cells show signs of activation [149, 179-181], which are higher in the liver than in the blood [180], and circulating MAIT cells are dysfunctional to TCR stimulation [180, 181]. Successful HCV-clearance therapy does not revert the decline in MAIT cells and their dysfunctionality in blood [86, 149, 180-182], but was reported to increase their levels and decrease their activation status in the liver [180]. In patients with HCV/HIV co-infections, MAIT cells in peripheral blood were detected at even lower levels than in HCV mono-infection alone [86, 179, 182]. In contrast, chronic hepatitis B virus (HBV) infection appears to only exert mild effects on MAIT cells, as recent studies found them not depleted in either the blood or in the liver of chronic HBV-infected patients [88, 183]. Moreover, circulating MAIT cells seem functionally intact, and their higher activation status when compared with healthy controls could be reversed by anti-viral therapy [183].

#### *1.2.9.5 Cancer, autoimmune diseases, and other clinical conditions*

The first studies examining MAIT cells in cancer reported the detection of the Vα7.2-Jα33 rearrangement in kidney and brain tumors [184], as well as in peripheral T cell lymphomas [185]. Subsequently, infiltration of MAIT cells in tumor tissues and metastases was reported in patients suffering from colon or colorectal cancer [186-190]. This indicates the capacity of

MAIT cells to infiltrate tumor tissues but further studies are warranted to ascertain their precise role in cancer development.

MAIT cells have also been studied in a variety of autoimmune diseases and other clinical conditions, such as Crohn's disease, ulcerative colitis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, asthma, obesity, juvenile type 1 diabetes, adult type 2 diabetes [reviewed in detail in 159, 191], and common variable immunodeficiency (CVID**)** [192]. Briefly, levels of circulating MAIT cells are decreased in patients with the aforementioned clinical conditions, except for children with type 1 diabetes or obesity, where circulating MAIT cell levels are reportedly unchanged or increased, respectively [159, 191, 192]. To date, investigation of MAIT cells in multiple sclerosis presents conflicting data [159, 191], and future studies are needed to clarify if and how their levels are affected in this disease.

Lastly, it should be noted that the precise role of MAIT cells in most of the aforementioned infections and clinical conditions (protective, pathogenic, or modulatory) remains to be determined, as most studies thus far have focused on their changes in frequency and on their functionality in peripheral blood and tissues.

## 1.3 HEPATITIS DELTA

The discovery of the hepatitis delta virus (HDV) goes back to 1977, when Rizetto *et al.* [193] detected a novel antigen, then named *delta antigen*, in hepatocytes from HBV-infected patients [193]. This antigen was initially considered a new biomarker of HBV infection [194], but was later on associated with an HBV-dependent RNA virus [195].

The HDV virion (35-37 nm in diameter) [195, 196] contains a single-stranded circular RNA genome that encodes only one protein, the *hepatitis delta antigen* (HDAg) [197-200]. HDV depends on host proteins for its replication and on HBV for its assembly, during which the HDV genome and HDAgs are enveloped by a lipid bilayer containing the HBV surface antigens (HBsAg) [195, 201, 202]. Therefore, hepatitis delta occurs in HBV-infected patients, and results from either simultaneous HBV-HDV co-infection of a naïve individual, or from HDV superinfection of a chronic HBV-infected patient [202, 203]. Similar to HBV, HDV is predominantly transmitted parenterally through contact with infected body fluids [202].

It is estimated that approximately 20 million people worldwide are infected with HDV [202, 204]. HBV/HDV co-infections cause the most severe form of viral hepatitis, and these patients have a higher risk of developing cirrhosis and hepatocellular carcinoma than HBV mono-infected patients [205, 206]. Although there is no established treatment for hepatitis delta, different therapeutic strategies based on the administration of pegylated-IFNα and HBV polymerase inhibitors are currently employed [202, 204]. HDV is a non-cytopathic virus [207], and the pathogenesis of hepatitis delta is believed to be mediated by the immune system [202, 204]. Despite this, both the innate and adaptive immune responses against HDV are defective. In fact, NK cells are functionally impaired [208, 209] and T cell responses are weak [210-213] during HDV infection.

# 2 AIMS

The overall aim of this thesis was to study the immunobiology of human MAIT cells and their functions in antimicrobial immunity. MAIT cells were relatively recently discovered, and the laboratory tools to specifically study these cells in humans - initially the monoclonal antibody against the highly conserved TCR Vα7.2 segment, and more recently the MR1 tetramers – became available during the last few 2 to 7 years. Given the novelty of these cells and tools, we first aimed to establish methodologies to specifically study MAIT cell functions *in vitro*. We then sought to investigate their responses to different stimuli and the possible compartmentalization of this cell population. Ultimately, we investigated if, how and why MAIT cells are affected in viral hepatitis infections in humans.

The specific aims of this thesis were to:

- Optimize and establish methodologies to study the diverse functions of MAIT cells, including activation, cytokine production, proliferation, degranulation, as well as their ability to kill target cells (**paper I**).

- Investigate the existence of potential heterogeneity within the MAIT cell population with regard to their surface immuno-proteome and their responses to TCR-dependent and –independent stimuli (**paper II**).

- Dissect the phenotypic and functional differences between $CD8^+$ and DN MAIT cells, and investigate the relationship between these two cell subsets (**paper III**).

- Determine the levels, phenotype, and functionality of MAIT cells in patients chronically infected with HDV, in comparison with chronic HBV mono-infected patients and healthy controls (**paper IV**).

# 3 METHODOLOGY

In this section, a summary of the main experimental approaches is presented. **Paper I** and **paper SIII** are method-dedicated articles that describe in detail methodologies optimized and established throughout the conduct of this thesis. The methods specifically used in each study are described in each paper.

The assays performed throughout this thesis can be broadly divided into *phenotypic* and *functional* experiments.

## 3.1 PHENOTYPIC EXPERIMENTS

These experiments aimed at determining the basal expression levels of surface and intracellular molecules in MAIT cells and other T cell populations. To this end, peripheral blood mononuclear cells (PBMCs) or cell suspensions obtained from tissues were stained with specific fluorochrome-labeled antibodies, and the expression levels were then determined by flow cytometry. Samples were acquired on an LSRFortessa flow cytometer (BD Biosciences) equipped with 355-, 405-, 488-, 561-, and 639-nm lasers, and they were analyzed using the FlowJo software version 9.8 or 9.9 (TreeStar).

## 3.2 FUNCTIONAL EXPERIMENTS

### 3.2.1 Experimental approaches

Functional experiments were conducted to study the activation, proliferation, and cytotoxicity of MAIT cells. They required either PBMC mixtures as the source of MAIT cells and APCs (Figure 5A), or magnetically purified $V\alpha7.2^+$ cells as the MAIT cell source, and autologous monocytes or the 293T cell line stably transfected with human MR1 (293T-hMR1 cells) as APCs (Figure 5B). In some functional experiments, pure MAIT cell populations obtained by fluorescence-activated cell sorting (FACS) were directly cultured with monocytes (Figure 5C). Microbes, either *E. coli* or *C. albicans*, were used as the source of MAIT cell antigens. In selected experiments, PBMCs were cultured with the cytokines IL-12 and IL-18 to assess MAIT cell activation in response to an innate cytokine stimulus (Figure 5A). The readouts of these assays (broadly presented in Table 1) were ultimately determined by flow cytometry using the same data acquisition instrument and analysis software as described in *Section 3.1*.

### 3.2.2 Selection of experimental approach

Specific experimental approaches were selected based on the type of assay to be performed and the type of biological material available. PBMC mixtures (Figure 5A) allowed for MAIT cell functions to be assessed in the presence of other cell types, and both direct and bystander activation of MAIT cells occurred in this system. It was particularly useful when using patient samples (**paper IV**), as the amount of cells was usually limited and further purification procedures would have resulted in significant cell loss.

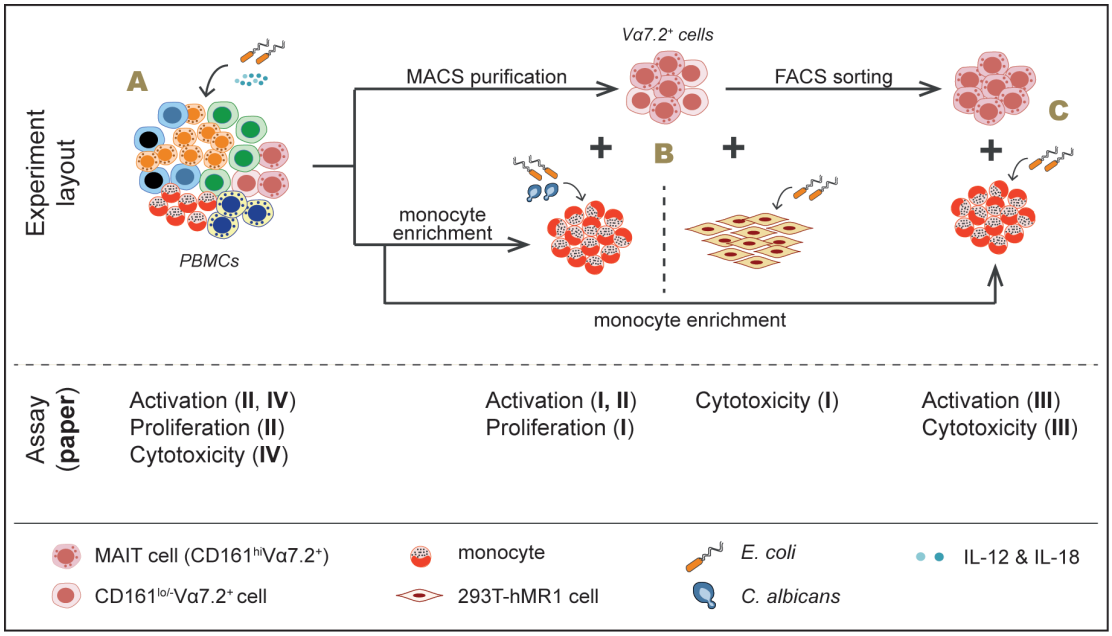


**Figure 5. Summary illustration of the functional assays performed throughout this thesis.** The papers where the assays have been used are indicated in brackets.

**Table 1. MAIT cell functional readouts.** Readouts assessed in MAIT cell analyses at the end of the activation, proliferation, and cytotoxicity assays. In some cytotoxicity assays, target cell death was also evaluated

| Assays | Surface readouts | Intracellular readouts |
|---|---|---|
| **Activation** | CD69<br>CD25<br>CD38<br>HLA-DR<br>PD-1<br>TIM-3 | IFNγ<br>TNF<br>IL-17 |
| **Proliferation** | | CTV dilution |
| **Cytotoxicity** | CD107a | GrzA<br>GrzB<br>Prf<br>Gnly |

Magnetic-activated cell sorting (MACS) of Vα7.2$^+$ cells (Figure 5B) (**paper I**) was suitable for obtaining a MAIT cell-enriched cell fraction for detailed studies of MAIT cell activation (**paper II**). In these assays, and due to the fact that Vα7.2$^+$ cells were obtained from peripheral blood, monocytes were selected as APCs (Figure 5B). As TCR-mediated activation of MAIT cells induces downregulation of the Vα7.2 segment (as well as of CD8), this co-culture system where Vα7.2$^-$ cells were virtually absent was particularly useful for the reliable identification of the MAIT cell population after activation (as discussed in *Section 5.1.4*). In this context, MAIT cell identification in stimulated PBMC mixtures (Figure 5A) was sometimes challenging, but this system was still deemed the best approach for studying patient material with low numbers of cells. Due to the simultaneous downregulation of CD8 during MAIT cell activation, PBMCs or total Vα7.2$^+$ cells could not be used to investigate

the functional differences between $CD8^+$ and DN MAIT cells. Thus, FACS sorting was conducted to obtain pure $CD8^+$ and DN MAIT cell populations, and these cells were then cultured with autologous monocytes (Figure 5C) (**paper III**).

In the cytotoxicity assays aimed at determining the capacity of MAIT cells to kill infected-target cells, 293T-hMR1 cells, which are relatively resistant to microbe-induced cell death, were used as APCs (Figure 5B) (**paper I**).

Proliferation assays were established with the $V\alpha7.2^+$-monocyte co-culture system (Figure 5B) (**paper I**) but were also carried out in PBMC mixtures (Figure 5A) when rare MAIT cell subpopulations defined by certain TCR Vβ segments needed to be analyzed (**paper II**).

### 3.2.3 Selection of MAIT cell stimulus

The MAIT cell activating antigens identified to date are intermediate metabolites of the riboflavin pathway [51, 52] (described in *Section 1.2.6.3*). This pathway is present in *E. coli* [52], and this bacterium was used as the natural source of MAIT cell agonists to establish methodologies and study MAIT cell functions throughout this thesis (**papers I-IV**). The opportunistic fungus *C. albicans*, which is also riboflavin biosynthesis-competent [52], was used in studies of microbe-dependent functional specialization of MAIT cell responses (**paper II**). The microbes were mildly fixed in formaldehyde before being used in the functional experiments to avoid microbial overgrowth during the time of culture. The innate, TCR-independent stimulation of MAIT cells was delivered through the combination of IL-12 and IL-18 (**papers II** and **IV**).

# 4 ETHICAL CONSIDERATIONS

All projects in this thesis were performed using human samples from healthy individuals and patients. Ethical approval was received from the appropriate Ethics Review Boards to conduct the experiments, and written informed consent was obtained from all donors. Detailed information about the type of samples and Ethics Review Boards is provided in the *Materials and Methods* section of each paper. However, it is noteworthy that all samples were de-identified in the laboratory setting. Additional information about the donors was sometimes provided depending on the type of sample and the purpose of the study, but that information did not lead to the identification of the donors.

In **papers I** to **IV**, we used peripheral blood from healthy donors, and had the possibility to request their gender and year of birth. In **paper III**, we additionally used endometrial samples and fetal spleens. Endometrial samples were accompanied with the donors' age, as well as with other information such as their menstrual status, hormonal treatment, and reason for surgery, as previously indicated [65]. For the fetal spleens, we obtained the gestational age [75] but no information about the mothers. In **paper IV**, we used peripheral blood from healthy controls, HBV mono-infected and HDV-infected patients, as well as liver biopsies from controls and HDV-infected patients. In addition to gender and year of birth, we obtained clinical data, including, among others, HBV and HDV viral load (as indicated in paper IV, Table 1). As all samples were de-identified, no experimental results were reported back to the respective donors.

# 5 RESULTS AND DISCUSSION

## 5.1 DEVELOPMENT OF METHODOLOGIES FOR MAIT CELL STUDIES

Adequate methodologies to study immune cell populations and their functions are essential in any area of immunology. In a relatively new field of research, such as that of MAIT cell studies, new and improved methodologies are crucial to advance knowledge, and their characterization and documentation provide the scientific community with basic protocols, which can be further adapted according to the questions being investigated. In **paper I**, we described in detail methodologies that we optimized and established to study MAIT cell effector functions *in vitro*, including activation, cytokine production, proliferation, cytotoxicity, and ability to kill target cells. These methodologies formed the basis of the experimental settings used in papers II and III.

The established methods rely on a co-culture system of peripheral blood Vα7.2$^+$ cells as the source of MAIT cells, and either monocytes (in activation and proliferation assays) or 293T-hMR1 cells (in cytotoxicity assays) as APCs. In contrast to PBMC mixtures, the composition of this system is well defined, which brings several advantages, as discussed in *Section 5.1.4*. In all assays, *E. coli* was used as the standard activating microbe and natural source of MAIT cell agonists.

Vα7.2$^+$ cells were MACS-sorted by positive selection from healthy individuals' PBMCs. Importantly, positive selection *per se* did not lead to activation of MAIT cells (**paper I**, Suppl. Fig. 1C), and MAIT cells retained a similar CD4/8 phenotype as that prior to purification (**paper I**, Suppl. Fig. 1B). This analysis was important to ensure that MAIT cells in the purified Vα7.2$^+$ cell fraction closely resembled those in the initial PBMC mixture, and that subsequent effector functions were not due to purification-driven activation of these cells.

### 5.1.1 Activation assay

In order to establish the activation assay, several technical parameters were optimized using CD69 upregulation concomitant with IFNγ production (CD69$^+$IFNγ$^+$) as functional readout for MAIT cell activation. The parameters optimized included microbial dose (*i.e.*, the *E. coli* colony-forming units (cfu):monocyte ratio), Vα7.2$^+$ cell:monocyte ratio, requirement of anti-CD28 as a co-stimulatory signal, and duration of the culture (**paper I**, Fig. 1). Furthermore, we tested different *E. coli* fixation times, and showed that mild fixation of *E. coli* resulted in similar levels of MAIT cell activation as with live *E. coli* (**paper I**, Fig. 1B-C). This observation justified the use of mild fixation in this type of assay, which is important to avoid overgrowth of microbes with short replication times, such as *E. coli*, during the experiment.

The optimized 24 h assay led us to look for other signs of MAIT cell activation. We detected upregulation of CD25 (**paper I**, Fig. 2A), and found that the simultaneous expression of CD69 and CD25 is more MR1-dependent than the expression of CD69 alone (**paper I**, Fig. 2B-C).

### 5.1.2 Proliferation assay

The proliferation assay was established using dilution of cell trace violet (CTV), a fluorescent proliferation-tracing reagent, in MAIT cells as functional readout. We optimized both the duration of the culture and the microbial dose, and found the 5-day assay to result in clear MAIT cell proliferation patterns that were predominantly MR1-dependent (**paper I**, Fig. 3). The detection of discernible CTV dilution peaks at the end of the assay can be further used to selectively study MAIT cells with different proliferation capacities. Of note, peripheral blood MAIT cells do not express Ki-67 at resting state [61, 144], and were initially reported to lack the capacity to proliferate *in vitro* [49, 61]. However several reports have since then demonstrated their capacity to upregulate Ki-67 following stimulation [144], and their ability to proliferate *in vitro* [57, 75, 144, paper I].

### 5.1.3 Cytotoxicity assay

The cytotoxicity assay was established in order to be able to evaluate the capacity of MAIT cells to degranulate and kill target cells. To this end, we used 293T-hMR1 cells as APCs (and target cells) because they are relatively resistant to *E. coli*-induced cell death (**paper I**, Suppl. Fig. 3G). We also pre-treated MAIT cells with IL-7 for 72 h, as this cytokine arms MAIT cells into $GrzB^{+}Prf^{+}$ cytolytic cells (paper SII and **paper I**, Suppl. Fig. 3A-B). We optimized the microbial dose (*i.e.*, the *E. coli* cfu:293T-hMR1 cell ratio), the effector MAIT cell:target 293T-hMR1 cell ratio, and the duration of the culture (**paper I**, Fig. 4).

Interestingly, this assay allowed us to distinguish between target cell apoptosis - as defined by positive staining of 293T-hMR1 cells for the fluorochrome-labeled inhibitor of caspases (FLICA; a reagent that labels cells undergoing caspase-mediated cell death), and by negative staining for the amine-reactive dead cell marker (DCM) (*i.e.*, $FLICA^{+}DCM^{-}$ cells) - and target cell full death defined as $FLICA^{+}DCM^{+}$. The former occurred within the first 6 h of culture, whereas the latter was detected following 24 h co-culture and coincided with MAIT cell degranulation, as evaluated by CD107a expression (**paper I**, Fig. 4E-F). Thus, this assay is particularly interesting as it allows the investigation of both the target cells and effector cells at the same time.

### 5.1.4 Advantages and limitations of the established methodologies

The assays established in **paper I** rely on well-defined co-culture systems, with a specific source of MAIT cells and MAIT cell agonists, and a defined type of APC. They are, therefore, highly versatile and can be adapted to study the effector functions of MAIT cells from tissues other than peripheral blood, the effect of APCs other than monocytes, and the

stimulatory capacity of microbes other than *E. coli*. Moreover, different components in the system can be blocked in order to investigate their respective involvement in the effector functions being studied. This includes not only MR1 and stress ligands in the target cells, but also receptors on MAIT cells with a yet unknown function, such as NKG2D.

When compared with experiments based on PBMC mixtures, the assays we established have the advantage of allowing for accurate flow cytometric identification of MAIT cells that have been activated or that proliferated in response to *E. coli.* TCR-mediated MAIT cell activation results in downregulation of CD3 and Vα7.2 in a microbial dose-dependent manner (Figure 6). Thus, accurate identification of MAIT cells by flow cytometry becomes challenging in stimulated PBMC mixtures as the MAIT cell population merges with $CD3^-$ and $V\alpha7.2^-$ cells (Figure 6). This problem is overcome in our system where $CD3^-$ and $V\alpha7.2^-$ cells are almost absent (Figure 6). On the other hand, proliferating MAIT cells in *E. coli*-stimulated PBMC mixtures downregulate CD161 (**paper I**, Suppl. Fig. 2A) as previously reported [144], and eventually merge with the few $CD161^-V\alpha7.2^+$ cells that have proliferated (**paper I**, Suppl. Fig. 2A). In contrast, we found that CD161 downregulation in our co-culture system is minor, with virtually no proliferation of $CD161^-V\alpha7.2^+$ cells (**paper I**, Suppl. Fig. 2A-B).

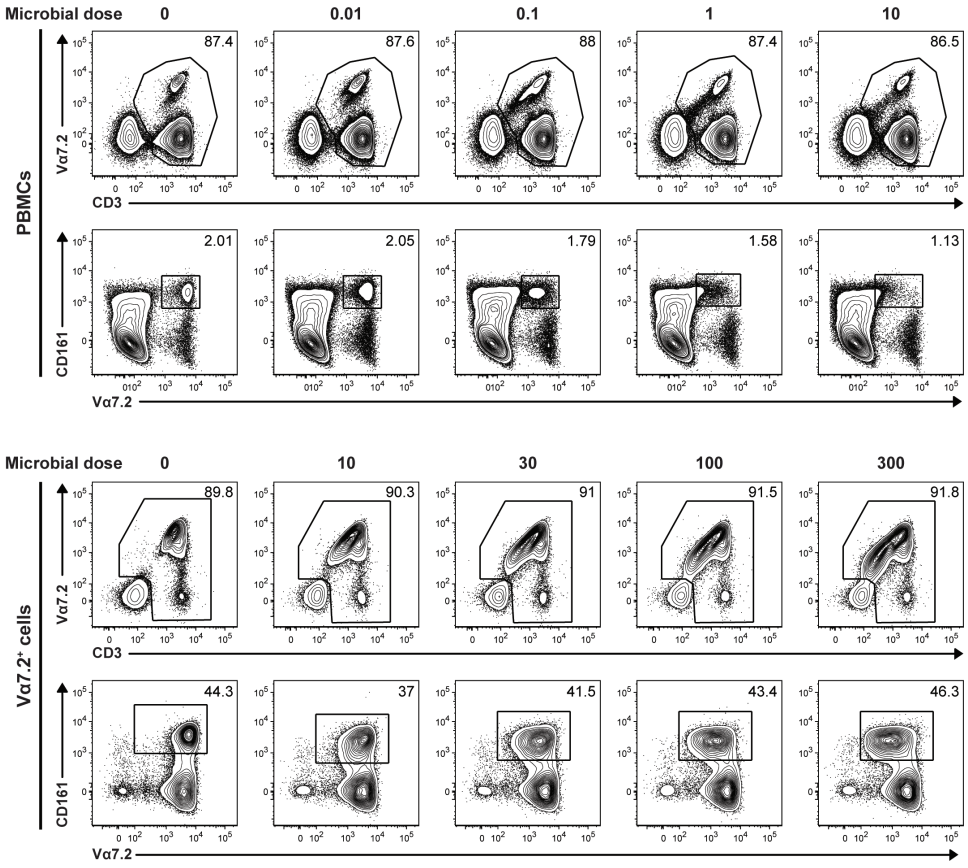


**Figure 6. Flow cytometric identification of $CD3^+$ cells and MAIT cells.** Stimulation of PBMCs (top) or $V\alpha7.2^+$-monocyte co-cultures (bottom) for 24 h with varying doses of formaldehyde-fixed *E. coli* results in strong downregulation of CD3 and TCR Vα7.2 on MAIT cells.

Besides these advantages, isolation of $V\alpha7.2^+$ cells and monocytes may be challenging when the goal is to study MAIT cells from patient samples, as the initial number of cells is already low and more purification procedures may lead to extensive cell loss. In these cases, the use

of mixed PBMC cultures is more appropriate. Overall, the assays described in **paper I** offer a valuable and versatile platform to study the immunobiology and functions of human MAIT cells in different immunologic contexts.

## 5.2 DIVERSITY OF MAIT CELL ANTIMICROBIAL RESPONSES

MR1 is highly evolutionarily conserved [41] and, to date, only a limited set of naturally occurring MR1-presented MAIT cell agonists, derived from the riboflavin biosynthesis pathway, has been identified [51, 52]. These facts have led to the notion that MAIT cells are a functionally homogeneous cell population, able to respond to riboflavin biosynthesis-competent microbes in an undifferentiated manner.

We investigated this hypothesis by studying the response of total MAIT cells to two highly distinct microbes, the bacterium *E. coli* and the opportunistic fungus *C. albicans* (**paper II**). When compared with *C. albicans*, *E. coli* induced significantly higher levels of IFNγ and TNF production by MAIT cells at the optimal microbial dose, as well as stronger downregulation of the Vα7.2 segment in a bacterial dose-dependent manner (**paper II**, Fig. 1A-D). These results identified *E. coli* as a potent MAIT cell activating microorganism, capable of eliciting a response pattern that may mimic that against potent pathogens. Furthermore, analysis of all combinations of IFNγ, TNF, and IL-17 production revealed distinct polyfunctional profiles, with MAIT cells responding to *E. coli* producing more IFNγ alone, TNF alone, a combination of IFNγ and TNF, or a combination of the three cytokines, compared with *C. albicans*-responding MAIT cells (**paper II**, Fig. 1E). Altogether, these results demonstrate the existence of microbe-specific MAIT cell response profiles.

MAIT cells, microbes, and APCs all play a role in MAIT cell responses, and it is reasonable to speculate that the functional outcome of microbial encounter by MAIT cells is ultimately determined by the interplay between all these components in the system. In *Sections 5.2.1* to *5.2.3*, the influence of each component in MAIT cell responses is discussed, and *Section 5.2.4* presents possible implications of the diversity of MAIT cell antimicrobial responses.

### 5.2.1 Characteristics of MAIT cells influencing their antimicrobial responses

#### *5.2.1.1 TCR β chain composition*

In an attempt to further understand factors that influence MAIT cells in their antimicrobial responses, we focused on their Vβ segment expression, which, although more restricted than that of other T cells [100, 119] (**paper II**, Fig. 2A and Suppl. Fig. 1-2), still adds some level of diversity to the MAIT cell TCR β chain repertoire. Overall, we found that the Vβ segment expression influenced MAIT cell responses to *E. coli* and *C. albicans*, as $V\beta8^+$, $V\beta13.1^+$, and $V\beta13.6^+$ MAIT cells produced less IFNγ and TNF in response to *E. coli* when compared with total MAIT cells (**paper II**, Fig. 2C-D and Suppl. Fig. 3A). In contrast, only $V\beta13.2^+$ MAIT cells responded slightly stronger than total MAIT cells to *C. albicans* (**paper II**, Fig. 2C-D

and Suppl. Fig. 3B). The fact that the Vβ bias differed between the responses to *E. coli* and *C. albicans* raises the interesting hypothesis that MR1 may present distinct antigens derived from these microbes.

Previous reports have supported the importance of the CDRα loops in the TCR interaction with MR1 [130, 132, 214], and, in particular, the role of the Y95 residue [51, 130, 131] (as described in *Section 1.2.6.4*), which is located in the CDR3α loop and is conserved among the Jα33, Jα12, and Jα20 sequences [53] (see *Section 1.2.6.2*). Overall, it is the TCR α chain that predominantly mediates the interaction between the MAIT cell TCR and MR1-ligand complexes [132, 214], and this may explain the high conservation of the Vα-Jα rearrangements characteristic of MAIT cells [132]. As for the β chain, individual residues within the CDRβ loops were reported to be dispensable for MR1 recognition [132]. However, several residues within the CDR2β and CDR3β loop were subsequently shown to interact with MR1 [130, 214], and it is thus possible that contacts established by the β chain overall fine-tune the TCR-MR1-ligand interaction. In this context, and in agreement with our results, López-Sagaseta *et al.* [131, 214] showed that MAIT cell TCRs carrying different Vβ segments bound bovine MR1, or humanized bovine MR1 in complex with a MAIT cell agonist with different affinities. Another study, however, showed that the interaction affinity was determined by the CDR3β loop rather than the Vβ segment itself [125]. Of note, only a limited set of TCRs were evaluated in this study, and the functional outcome was assessed using TCR-transfected SKW3 cells and C1R.MR1 cells pre-incubated with synthetic ligands [125]. In contrast, our assay allowed screening of a much larger number of TCRs in a naturally occurring MAIT cell population responding to a natural source of MR1 ligands.

We also assessed the influence that Vβ segment expression has on the proliferative capacity of MAIT cells. Following a five-day culture of CTV-labeled PBMCs in the presence of *E. coli* and IL-2, we found that the initially less abundant Vβ-defined MAIT cell subpopulations were less proliferative *in vitro* (**paper II**, Fig. 3). In fact, there was an inverse correlation between the initial frequency of Vβ-defined MAIT cell subpopulations in the PBMC mixture and the CTV geometric MFI in these subpopulations after five days of culture (**paper II**, Fig. 3B). These findings raise the possibility that the *in vivo* interaction of MAIT cells with microbes, such as those that compose the microbiota, may overall shape the Vβ repertoire of MAIT cells, as cells carrying more responsive TCRs may expand to a greater extent than less responsive ones. Our data on the differential responsiveness of Vβ-defined MAIT cell subpopulations (**paper II**, Fig. 2C-D and Suppl. Fig. 3) are consistent with this notion. Interestingly, and in light of this hypothesis, one can speculate that the MAIT cell Vβ repertoire will differ between individuals with distinct features known to affect the microbiota, such as geographic location, diet, and use of medication [215, 216]. In a recent study, Hinks *et al.* [95] reported that the levels of MAIT cells were decreased in the peripheral blood and bronchial tissue of steroid-treated patients with chronic obstructive pulmonary disease (COPD), in comparison with non-treated patients. Moreover, corticosteroids negatively affected MAIT cell antibacterial responsiveness *in vitro* [95]. Whether this or any other of the aforementioned factors contribute to the shaping of the

MAIT cell Vβ repertoire through their effect on the microbiota composition is currently unknown. This question could potentially be addressed by studying MAIT cells in cohorts of individuals that have been previously used in extensive microbiota studies [215, 216].

In summary, we conclude that the TCR β chain has some influence on the MAIT cell recognition of MR1-ligand complexes. Thus, the relative abundance of different Vβ-defined MAIT cell subpopulations, which may have already been determined by microbial encounter *in vivo*, may shape the MAIT cell responses to the same microbe as well as distinct microbes.

#### *5.2.1.2 CD8 co-receptor expression*

Given that $CD8^+$ and DN MAIT cells represent the majority of circulating MAIT cells in healthy adults [50, 63], we investigated the responsiveness of these subsets to *E. coli* stimulation *in vitro* (**paper III**, Fig. 3). Previous studies have evaluated responses of these MAIT cell subsets to *H. pylori* [97] and PMA/ionomycin [217]. However, because these experiments were conducted using PBMC mixtures [97, 217] and the CD8 molecule is partly downregulated upon activation (**paper III**, Suppl. Fig. 2A), it is possible that stimulated DN MAIT cells in these experiments represented a mixture of *bona fide* DN MAIT cells, and $CD8^+$ MAIT cells that have downregulated CD8 following activation. In our experiments, we first FACS-sorted $CD8^+$ and DN MAIT cells (Figure 5C), and then separately stimulated these purified MAIT cell subsets. We found that $CD8^+$ MAIT cells responded more strongly to *E. coli*, with significantly higher production of IFNγ, TNF, and GrzB than DN MAIT cells (**paper III**, Fig. 3). The superior responsiveness of $CD8^+$ MAIT cells was consistent with their higher expression levels of CD2 and CD9 **(paper III**, Fig. 1B-C and Suppl. Table 1), both T cell co-stimulatory molecules [218, 219], the cytotoxic molecules GrzB, Prf, and Gnly (**paper III**, Fig. 1B-C), and the transcription factors T-bet and Eomes (**paper III**, Fig. 2).

In classical models of T cell activation, TCR engagement induces the recruitment and clustering of the TCR/CD3 complexes to specific cell membrane domains with a distinct molecular composition known as *rafts* or *detergent insoluble glycolipid-enriched* (DIG) *fractions* [220, 221]. The TCR/CD3 complex recruitment is accompanied by accumulation of signal-transducing substrates and enzymes in the DIG fractions, and results in early downstream TCR signaling cascades, which lead to T cell activation [220, 221]. Yashiro-Ohtani *et al.* [222] showed that CD2, CD5, and CD9 are present in the DIG fractions, and that they exert their T cell co-stimulatory effects by enhancing the association between the TCR/CD3 complexes and these fractions [222]. Although we did not detect any major differences in the expression of CD5 between $CD8^+$ and DN MAIT cells (**paper III**, Suppl. Table 1), it is plausible that the higher surface expression of CD2 and CD9 by $CD8^+$ MAIT cells may partly explain their stronger responses to TCR stimulation.

The higher GrzB content in resting $CD8^+$ MAIT cells (**paper III**, Fig. 1B-C), although low when compared with stimulated cells, is consistent with their superior capacity to produce GrzB following *E. coli* stimulation (**paper III**, Fig. 3). This, combined with higher basal levels of Prf could possibly translate into a superior killing capacity of $CD8^+$ MAIT cells

when compared with their DN counterparts. In an interesting study where TCR αβ transgenic mice were used to compare the functionality of positively selected αβ $CD8^+$ T cells and non-positively selected αβ DN T cells, Caveno *et al.* [223] showed that $CD8^+$ T cells were more efficient in killing target cells, and they were also superior in antigen-driven proliferation and IL-2 production when compared with their negative counterparts (although IFNγ production was similar between subsets) [223]. While it is tempting to speculate that a similar cytolytic bias for human $CD8^+$ MAIT cells may exist, cytolytic assays, potentially similar to those established in paper I, are required to truly compare the killing capacity of $CD8^+$ and DN MAIT cells.

It is currently not known why $CD8^+$ MAIT cells are functionally superior to DN MAIT cells. In conventional $CD8^+$ T cells, CD8 binds the α3 domain of the MHC class I molecule, thereby increasing the avidity of the $CD8^+$ T cell-APC interaction [224, 225]. In a similar manner, it is possible that CD8 stabilizes the interaction between the MAIT cell TCR and the MR1-ligand complex, leading to stronger $CD8^+$ MAIT cell responses. Consistent with this hypothesis, CD8 blockade was shown to decrease MAIT cell responses to *E. coli* [226]. Interestingly, however, it differently affected MAIT cell functional readouts, with the production of IFNγ and TNF decreasing more than degranulation upon CD8 blocking [226]. In a similar pattern, Caveno *et al.* [223] showed that CD8 blockade decreased the proliferation capacity of $CD8^+$ T cells to levels similar to that of DN T cells, whereas the killing efficiency and IL-2 production were not affected to the same extent [223]. Altogether, these studies suggest that while direct CD8 binding to MR1 may influence $CD8^+$ MAIT cell effector functions, other cell intrinsic or environmentally driven mechanisms, which remain to be determined, may also be involved. Of note, because the use of a CD8 blocking antibody may have secondary effects in the assays, such as preventing the interaction between the TCR and the antigen-presenting molecule, experiments where either potential CD8 binding sites in MR1 are disrupted or the *CD8* gene is deleted through the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas 9) genome editing platform would be important to further investigate the effect of CD8 in MAIT cell functions [226].

Stimulation of $CD8^+$ and DN MAIT cells with PMA/ionomycin showed a similar pattern of responses, with $CD8^+$ MAIT cells producing IFNγ and TNF at higher levels than DN MAIT cells (**paper III**, Fig. 3). Interestingly, however, DN MAIT cells produced significantly more IL-17, although its levels were much lower than those of IFNγ or TNF (**paper III**, Fig. 3). A recent study on patients with primary Sjögren's syndrome where MAIT cells are polarized towards IL-17 production has shed light on the cellular pathways leading to IL-17 production [227]. These involved IL-23 and IL-7, which induced upregulation of RORc, or STAT3 and HIF1α transcripts, respectively, ultimately leading to IL-17 production [227]. It is thus possible that these pathways are overrepresented in peripheral blood DN MAIT cells.

IL-17 is a pro-inflammatory cytokine that plays a protective role against infections by several species of bacteria and fungi [228, 229]. Interestingly, it is essential in protection against *C.*

*albicans*, as IL-17R deficient mice were reported to be highly susceptible to this pathogen and eventually succumbed to the infection [230]. Whether DN MAIT cells also produce more IL-17 than $CD8^+$ MAIT cells in response to *C. albicans* has not been evaluated. However, one can speculate that DN MAIT cells may contribute with IL-17 production to the overall MAIT cell antimicrobial response, adding on to Th1 cytokines and cytotoxic molecules produced at higher levels by $CD8^+$ MAIT cells. Of note, and similar to other pro-inflammatory cytokines, excess IL-17 contributes to pathology and tissue damage, and it is the balance in the levels of these different pro-inflammatory mediators that determines the outcome of the immune response to a specific pathogen (*i.e.*, protective *vs.* pathogenic) [228, 229].

Overall, we can conclude that $CD8^+$ MAIT cells display superior functionality to TCR and mitogen stimulations, and the relative abundance of $CD8^+$ MAIT cells may, therefore, also shape MAIT cell antimicrobial responses. Importantly, we have not directly assessed the responsiveness of purified $CD8^+$ and DN MAIT cells to *C. albicans*. However, if the higher responsiveness of $CD8^+$ MAIT cells is predominantly dictated by CD8 binding to MR1, it is likely that we would obtain similar results with *C. albicans*. Nonetheless, similar activation experiments are required to confirm this hypothesis. In addition, it would be interesting to evaluate other antimicrobial functions besides activation and cytokine production in order to ascertain whether the superior functionality of $CD8^+$ MAIT cells is maintained throughout other effector functions.

$CD4^+$ MAIT cells represent only a minor subset of total MAIT cells, and only approximately one third of this subset defined by the expression of Vα7.2 and high levels of CD161 stains with the MR1 5-OP-RU tetramer (**paper III**, Fig. 1A), in agreement with previous reports [53, 226]. Kurioka *et al.* [226] reported marked phenotypic and functional differences between $CD4^+$ MAIT cells and the other two subsets [226]. Upon *E. coli* stimulation, and in contrast to $CD8^+$ and DN MAIT cells, $CD4^+$ MAIT cells produced less cytotoxic molecules and Th1 cytokines but more Th2 cytokines than $CD8^+$ and DN MAIT cells [226]. However, these results should be carefully interpreted as $CD4^+$ MAIT cells were identified in these specific experiments based on CD161 and Vα7.2 co-expression [226], and the presence of non-MR1 restricted T cells, possibly not responsive to *E. coli*, may underestimate the overall functionality of *bona fide* $CD4^+$ MAIT cells.

#### *5.2.1.3 Conclusions on the characteristics of MAIT cells influencing their antimicrobial responses*

In summary, we show that MAIT cell TCR-mediated responses may be influenced by two factors intrinsic to the MAIT cells themselves: the TCR β chain composition and CD8 expression. Vβ-defined MAIT cell subpopulations are associated with different degrees of responsiveness to microbial stimulation, and $CD8^+$ MAIT cells display higher functional capacity than DN MAIT cells both to microbial and mitogen stimulations.

These independent observations led us to evaluate the relationship between Vβ segment expression and CD8 expression on MAIT cells. While we showed that $CD8^+$ MAIT cells were superior in their *in vitro* responsiveness when compared with DN MAIT cells (**paper III**, Fig. 3), direct comparison of the abundance of Vβ-defined subpopulations with different degrees of functionality between $CD8^+$ and DN MAIT cells has not been previously performed. Strikingly, this analysis showed that the *E. coli*-hyporesponsive $V\beta13.1^+$ and $V\beta13.6^+$ MAIT cells were significantly more abundant in $CD8^+$ MAIT cells, as were $V\beta7.2^+$ MAIT cells for which we found no differences in functional capacity in comparison with total MAIT cells (**paper II**, Fig. 2D and Suppl. Fig. 3) (Figure 7). As $V\beta13.1^+$ and $V\beta13.6^+$ MAIT cells cover less than 8% of the total $CD8^+$ MAIT cell population (**paper III**, Fig. 5C), one can speculate that the predominance of other responsive Vβ-defined MAIT cells may overcome the lower functional capacity of these two Vβ-defined subpopulations in $CD8^+$ MAIT cells. It should also be noted that there are other Vβ-defined MAIT cell subpopulations for which we did not assess the *in vitro* functionality (**paper III**, Fig. 5C), and these will contribute to shape the overall functional capacity of $CD8^+$ and DN MAIT cells.

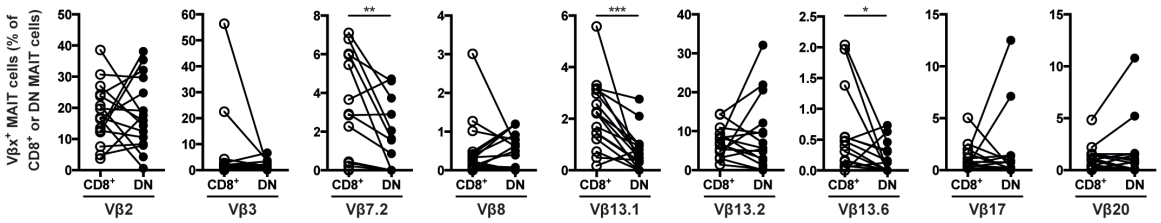


**Figure 7. Frequency of Vβ-defined MAIT cell subpopulations in resting $CD8^+$ and DN MAIT cells.** Data are from 14 to 16 donors. Lines represent individual donors. The Wilcoxon's test or paired *t* test was used to detect significant differences between the paired samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Given the differences in functionality dictated by both the TCR β chain and CD8 expression in peripheral blood MAIT cells, one could expect accumulation of non-hyporesponsive Vβ-defined subpopulations and highly functional $CD8^+$ MAIT cells at sites where microbial encounter is more likely to occur, such as the liver and the gut, to benefit the host. In particular, the presence of $V\beta13.2^+$ MAIT cells, which cover a significant proportion of the MAIT cell population (**paper II**, Fig. 2A and **paper III**, Fig. 5C), could potentially boost anti-*C. albicans* immune responses at sites of colonization, such as the genitourinary and oropharyngeal tracts [231]. While the Vβ repertoire has not been analyzed in MAIT cells from sites other than the blood, $CD8^+$ MAIT cells from rectal mucosa were shown to express higher levels of genes associated with activation and pro-inflammatory functions, including *TNF*, *IL-23R*, and *CD40L*, than their negative counterparts [153]. In another study, liver $CD8^+$ MAIT cells were the main producers of IFNγ within the MAIT cell population in response to TLR8 agonist stimulation of hepatic cells [88]. More studies dissecting the composition of tissue MAIT cells in terms of their Vβ segment and CD8 expression would be important in this context.

Overall, we can conclude that both the TCR β chain composition and CD8 expression affect the type and magnitude of peripheral blood MAIT cell effector functions, and contribute to the functional heterogeneity they display in their array of antimicrobial responses (Figure 8).

### 5.2.2 Influence of microbial characteristics on MAIT cell responses

#### *5.2.2.1 Production of MAIT cell antigens and other microbial products*

The riboflavin biosynthesis pathway, present in many species of bacteria and fungi [121, 122], is the only natural source of MAIT cell activating antigens identified so far (see *Section 1.2.6.3*). This pathway is present in both *E. coli* and *C. albicans* [52], but diverse factors may influence their capacity to activate MAIT cells and contribute to the differential responses detected (**paper II**, Fig. 1A-E).

The type and concentration of MAIT cell agonists and antagonists likely depend on the type of microbe and on its growth phase. The most potent agonist identified to date, 5-OP-RU, requires 5-A-RU and either glyoxal or methylglyoxal for its formation [51] (see *Section 1.2.6.3*). The local concentration of these precursors may, therefore, dictate the amount of 5-OP-RU generated [107]. The strong Vα7.2 downregulation detected following MAIT cell activation with *E. coli* (**paper II**, Fig. 1C) suggests that this microbe may produce more potent or more abundant MAIT cell agonists. On the other hand, the natural MAIT cell antagonists derive from folic acid acquired from the diet [52, 125, 126] (see *Section 1.2.6.3*), and the concentration of folic acid at MAIT cell effector sites might also determine the amount of antagonists locally available. Of note, it is clearly possible that other, still-unidentified MAIT cell antigens exist with other requisites for their formation. Overall, the fine balance between the type and concentration of agonists and antagonists will likely influence the MAIT cell functional outcome upon microbial encounter, similar to what has been shown in *in vitro* competition experiments between MAIT cell agonists and antagonists [125, 126, 129].

Interestingly, there is also the possibility that other microbial products interfere with MAIT cell responses without binding to MR1. Such an hypothesis was proposed in a recent study where lactate added to cultures partially mimicked the effect of *Lactobacilli*-derived cell free supernatant in decreasing the activation of NK and T cells in response to *S. aureus* [232]. These findings suggested an immunomodulatory role for lactate in lymphocyte activation [232]. Other studies have demonstrated that short chain fatty acids that are produced during bacterial fermentation, including acetate, propionate, and butyrate, promote T cell differentiation into Treg cells, as well as into Th1 or Th17 cells depending on the cytokine milieu [233, 234]. Thus, it is plausible that, when locally released, these types of compounds may affect MAIT cell responses to the microbes producing them as well as to adjacent microbes.

#### *5.2.2.2 Differential propensity for phagocytosis and PAMP repertoire*

Geometric parameters, such as size and shape, can influence phagocytosis of particles [235]. The microbes *E. coli* and *C. albicans* are very distinct in size and morphology. *E. coli* is rod-shaped [236], whereas *C. albicans* is a bigger microbe that can grow as unicellular yeast, filamentous hyphae, or filamentous pseudohyphae [237]. Thus, one can assume that the strikingly different physical properties of *E. coli* and *C. albicans* will influence their propensity to be phagocytosed, and ultimately their abundance (or intracellular microbial load) in the APCs. Salerno-Goncalves *et al.* [134] showed, using a B cell line as APC and *Salmonella enterica* serovar Typhi and *E. coli* as microbes, that the quality of the MAIT cell response (*i.e.*, the type of cytokines produced) may depend on the bacterial load [134]. This raises the interesting hypothesis that MAIT cell responses to *E. coli* and *C. albicans* may also be shaped by the intracellular concentration of these microbes.

Given the highly distinct nature of these microbes, the PAMPs expressed on their surface are likely to be different and may trigger different TLRs on the APCs. Exposure to different TLR agonists in *E. coli*-stimulated cultures was shown to positively or negatively influence MAIT cell IFNγ production [113]. Thus, the PAMP-TLR interactions are likely to represent another factor through which MAIT cell antimicrobial responses may be shaped.

#### *5.2.2.3 Conclusions on the influence of microbial characteristics on MAIT cell responses*

Overall, we can conclude that a wide array of microbe-intrinsic factors may shape MAIT cell effector functions, ranging from their physical properties and surface phenotype to the type and abundance of MAIT cell antigens and other microbial products (Figure 8). The combination and interplay between all these factors is also likely to influence the amount of microbe that is necessary for optimal MAIT cell responses. While we have already reported that *E. coli* induced significantly higher levels of IFNγ than *C. albicans* (**paper II**, Fig. 1A-B, D), it is noteworthy here that the optimal dose of *E. coli* required to reach maximal MAIT cell activation, as assessed by the upregulation of CD69 and production of IFNγ, was much higher than that of *C. albicans* (**paper II**, Fig. 1B).

### 5.2.3 Influence of APC characteristics on MAIT cell antimicrobial responses

#### *5.2.3.1 Repertoire of co-signaling receptors*

The MAIT cell effector functions against *E. coli* and *C. albicans* (**paper II**, Fig. 1-2) were studied using monocytes as APCs (**paper I**, Fig. 1). In our experimental system, we found that the addition of anti-CD28 in the monocyte-Vα7.2$^{+}$ cell co-cultures stimulated with *E. coli* boosted MAIT cell IFNγ production (**paper I**, Fig. 1E). This indicates that monocytes are not intrinsically very efficient in delivering co-stimulatory signals, and, importantly, that the magnitude of the MAIT cell response depends to some degree on the level of co-stimulation provided by the APC. Moreover, *E. coli* stimulation of MAIT cells induced only modest upregulation of the co-inhibitory receptor T-cell immunoglobulin and mucin domain-containing protein-3 (TIM-3) (**paper I**, Fig. 2A). Overall, and in a similar manner to

conventional T cells [238], it is likely that the repertoire of co-stimulatory and co-inhibitory receptors expressed by the APC will influence both the type and magnitude of MAIT cell responses.

#### *5.2.3.2 MR1 antigen presentation*

MR1 is mostly kept intracellularly at resting state and it traffics to the cell surface in a process dependent on APC activation (see *Section 1.2.6.1*). In our activation experiments, MR1 blocking led to discrepant results with regard to the production of IFNγ and TNF. IFNγ production was mostly MR1-dependent, but a certain amount of this cytokine was still produced independently of the TCR-MR1 interaction (**paper II**, Fig. 1F-G). In contrast, the MR1-dependency of TNF production was significantly higher than that of IFNγ, and almost no TNF was detected upon MR1 blocking (**paper II**, Fig. 1F-G). These results suggest the existence of variable requirements for production of different cytokines in MAIT cells. The dependency on TCR-MR1 interaction for the production of virtually all of the TNF and most of IFNγ shows that MAIT cell pro-inflammatory responses are tightly regulated. This may be particularly important in order to avoid such responses against riboflavin biosynthesis-competent microbes from the microbiota, which may be in close proximity to MAIT cells but not actively producing MAIT cell agonists at steady state. Of note, the fact that MR1 is not constitutively expressed on the APC surface at steady state may also be considered a MAIT cell activation regulatory mechanism. As it was shown that MR1 can bind extracellular ligands directly on the cell surface [114] (see *Section 1.2.6.1*), the existence of potential MR1 ligands from commensal microbes, even if at homeostatic levels, could otherwise result in unnecessary MAIT cell pro-inflammatory responses.

#### *5.2.3.3 Production of innate cytokines*

As microbes activate APCs not only to upregulate co-stimulatory molecules but also to secrete cytokines, such as IL-12 and IL-18 [1], the MR1-independent MAIT cell IFNγ production may be due to the direct stimulatory effect of monocyte-derived cytokines on MAIT cells. Given that MAIT cells express IL-12R and IL-18R [49, 70, 71], it is plausible to assume that these cytokines contribute to MR1-independent activation of these cells upon microbial encounter. In **paper II** (Fig. 5D-E), we showed that $CD56^+$, $CD84^+$, and $CD94^+$ MAIT cells display a higher capacity to respond to innate cytokines than their negative counterparts. Thus, the relative proportion of these MAIT cell subsets might influence the overall MAIT cell antimicrobial responses. As MAIT cell responses against viruses are probably primarily driven by innate cytokines (see *Section 1.2.7.5*), these findings are discussed in more detail in *Section 5.4.1*, in the context of MAIT cell involvement in viral hepatitis.

#### *5.2.3.4 Conclusions on the influence of APC characteristics on MAIT cell antimicrobial responses*

In conclusion, several aspects of APCs may shape MAIT cell antimicrobial responses, including the repertoire of co-stimulatory and co-inhibitory receptors, the surface expression

of MR1-antigen complexes, and the innate cytokines they produce upon microbial exposure (Figure 8).

Given the ubiquitous expression of MR1 [40, 47, 110], many cell types apart from monocytes can present antigens to MAIT cells (see second paragraph of *Section 1.2.7.3*). Professional APCs, namely DCs, macrophages, and B cells, are capable of microbe internalization and intracellular processing, as well as of delivering co-stimulatory signals to T cells [1]. In addition, APCs produce different types of innate cytokines upon stimulation [239], and the occurrence and extent of MR1 upregulation varies among cell types [113]. As all of these factors vary with the type of APC, they will likely also influence the type and magnitude of MAIT cell responses. In antimicrobial responses *in vivo*, where MAIT cells are more likely to respond to a mixture of different microbe-exposed APCs, the panel of MAIT cell responses may be ultimately influenced by the type and relative abundance of each APC in the system.

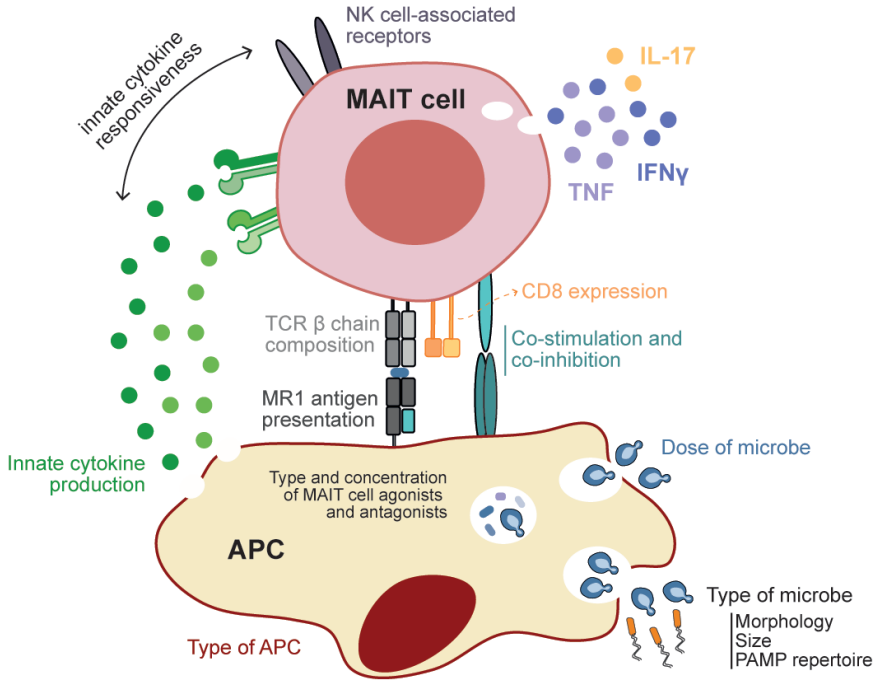


**Figure 8. Summary illustration of the characteristics of MAIT cells, microbes, and APCs that may influence MAIT cell antimicrobial responses.**

### 5.2.4 Implications of the diversity of MAIT cell antimicrobial responses

Overall, MAIT cell responses to distinct microbes are diverse, and we identified several factors influencing the quality and magnitude of the responses. These include the microbial dose, the TCR interaction with MR1, as well as the TCR β chain composition and the expression of CD8 and NK cell-associated receptors on MAIT cells. The concept of functional heterogeneity/compartmentalization has also been shown for conventional $CD4^+$ and $CD8^+$ T cells [98, 240, 241]. Strategic location of these different T cell populations at sites of microbial encounter combined with their capacity to recognize different types of antigens is likely to benefit the host, as these cells help form multifaceted immune barriers for immunosurveillance and defense against microbial invaders.

MAIT cells represent a significant fraction of the T cell compartment in peripheral blood and in the gut, and even more so in the liver (see *Section 1.2.5*). Factors underlying their functional heterogeneity and the immunobiology behind their potent effector responses, which we are just now beginning to understand, may be harnessed in the future in the development of immunotherapeutic approaches for the treatment of a wide variety of diseases. Current immunotherapies include infusion of lymphocytes as well as administration of cytokines to augment or dampen pro-inflammatory responses [242-245]. The development of MAIT cell-directed therapies should benefit from the knowledge generated in this field and, if proven successful, these therapies could potentially be combined with existing strategies to ultimately improve treatments and disease outcomes.

## 5.3 RELATIONSHIP BETWEEN CD8$^+$ AND DN MAIT CELLS

Although there has been much attention focused on the biology of MAIT cells and their role in the immune system, the relationships between MAIT cell subsets has been largely unexplored. As CD8$^+$ and DN MAIT cells represent the vast majority of MAIT cells, unraveling the relationship between these two cell subsets will be important for our understanding of MAIT cell immunobiology.

### 5.3.1 Potential transition from CD8$^+$ MAIT cells to DN MAIT cells

Our analysis of the Vβ segments of adult peripheral blood CD8$^+$ and DN MAIT cells revealed that CD8$^+$ MAIT cells expressed a significantly more diverse Vβ repertoire than their DN counterparts (**paper III**, Fig. 5C-D). Strikingly, the Vβ segments detected in DN MAIT cells were also present in CD8$^+$ MAIT cells, but not vice-versa. This was initially observed when all donors were analyzed collectively (**paper III**, Fig. 5C). However, the same pattern occurred in each individual donor (**paper III**, Suppl. Fig. 3B), which supports the idea that, in any given individual, the Vβ repertoire of DN MAIT cells *in vivo* is a subset of that of CD8$^+$ MAIT cells. This finding, together with the fact that CD8 is downregulated from the surface following MAIT cell activation (**paper III**, Suppl. Fig. 2A), suggests that DN MAIT cells may represent a subset of MAIT cells that were originally CD8$^+$ but have downregulated the CD8 co-receptor upon activation *in vivo*.

This hypothesis is consistent with our data on second-trimester fetal CD8$^+$ and DN MAIT cells (**paper III**, Fig. 5A-B), which we obtained by directed analysis of CD8$^+$ *vs.* DN MAIT cells in human fetal tissues [75]. This analysis revealed that fetal DN MAIT cells were more mature than CD8$^+$ MAIT cells, as the former expressed significantly higher levels of CD45RO and IL-18R, and lower levels of CD62L and CCR7 (**paper III**, Fig. 5B). Moreover, during gestational development, the frequency of DN MAIT cells in the CD4$^-$ MAIT cell compartment increased over time, with a corresponding contraction of the CD8$^+$ MAIT cell population (**paper III**, Fig. 5A). This suggests an accumulation of DN MAIT cells during fetal development. In agreement with this, Koay *et al.* [63] recently reported that DN MAIT

cells are rare in the thymus but more abundant in young peripheral blood and even more so in adult peripheral blood [63]. Collectively, our data on fetal MAIT cells and on the Vβ repertoire of adult peripheral blood MAIT cells suggest that DN MAIT cells may derive from the larger $CD8^+$ MAIT cell population *in vivo*.

In the original study on fetal MAIT cells [75], the combined evaluation of the expression of PLZF, CD62L, and CD45RO showed that fetal CD8αα MAIT cells were more mature than $CD8\alpha\beta^{lo}$ MAIT cells, whereas $CD8\alpha\beta^{hi}$ MAIT cells were essentially naïve [75]. This led to the hypothesis that fetal CD8αα MAIT cells could be derived *in vivo* from CD8αβ MAIT cells in a stepwise $CD8\alpha\beta^{hi}$ → $CD8\alpha\beta^{lo}$ → CD8αα manner [75]. A similar pattern was suggested by Walker *et al.* [62] for adult peripheral blood $CD161^{hi}CD8^+$ T cells, as $CD161^{hi}$CD8αα T cells could be derived *in vitro* from $CD161^{hi}$CD8αβ T cells [62], similar to what occurs with conventional $CD8^+$ T cells [246]. Consistent with these findings, $CD8^+$ MAIT cells in the thymus and cord blood are exclusively CD8αβ [62, 63] (see *Section 1.2.4*), whereas those in peripheral blood express CD8αα at gradually increasing levels from young to adult peripheral blood [63].

In summary, our data, together with previous findings, suggest that DN MAIT cells may derive from $CD8^+$ MAIT cells in the following stepwise model: CD8αβ MAIT → CD8αα MAIT → DN MAIT cells.

### 5.3.2 Potential transition from DN MAIT cells to cell death

Further analysis of the MAIT cell subsets revealed that DN MAIT cells were more prone to apoptosis than $CD8^+$ MAIT cells both at resting state and following *E. coli* or PMA/ionomycin stimulations (**paper III**, Fig. 4A-B), as assessed by their higher staining for FLICA (**paper III**, Fig. 4A-B). These results are consistent with the higher expression levels of PLZF in adult and fetal DN MAIT cells (**paper III**, Fig. 2 and Fig. 5B, respectively), which was previously shown to drive the pro-apoptotic features of MAIT and iNKT cells [247]. In agreement with these findings, Kurioka *et al.* [226] have recently reported higher levels of Annexin V in DN MAIT cells following *E. coli* stimulation [226]. Of note, the higher propensity of DN MAIT cells for apoptosis occurred despite similar expression levels of the anti-apoptotic protein Bcl-2 [1, 248] between $CD8^+$ and DN MAIT cells (**paper III**, Fig. 4D). It would be important, nonetheless, to investigate the expression levels of other anti-apoptotic proteins, such as Bcl-$X_L$, as well as of pro-apoptotic proteins, such as the Bcl-2 associated X, apoptosis regulator (Bax) and the Bcl-2 antagonist/killer (Bak), as it is the overall balance between these proteins that helps dictate the fate of a cell [1, 248]. Moreover, it would be interesting to evaluate the expression levels of the X-linked inhibitor of apoptosis (XIAP), which was shown to counteract the pro-apoptotic phenotype induced by PLZF on MAIT and iNKT cells [247].

Given the higher propensity of DN MAIT cells for apoptosis, and in light of the results presented in *Section 5.3.1*, we suggest an irreversible transition from $CD8^+$ MAIT cells to DN MAIT cells to eventual cell death. This hypothesis could potentially explain the lower

functionality of DN MAIT cells when compared with $CD8^+$ MAIT cells (**paper III**, Fig. 3) (discussed in *Sections 5.2.1.2* and *5.2.1.3*), as these cells are progressing to cellular senescence and eventually cell death. Interestingly, in the aforementioned murine study on positively selected αβ $CD8^+$ T cells and non-positively selected αβ DN T cells [223], Caveno *et al.* reported that DN T cells isolated from antigen-exposed mice were functionally anergic, as suggested by their poor proliferation and low IL-2 production following antigen stimulation *in vitro* (even though no comparison was made with $CD8^+$ T cells) [223].

### 5.3.3 Conclusions on the relationship between $CD8^+$ and DN MAIT cells

Altogether, we suggest the following model summarizing the derivative relationships between MAIT cell subsets: CD8αβ MAIT → CD8αα MAIT → DN MAIT → cellular senescence → cell death. In agreement with this, while $CD8^+$ MAIT cells were detected at lower levels in the peripheral blood of chronic HCV-infected patients when compared with healthy controls, the levels of DN MAIT cells remained relatively unchanged [180]. This is consistent with a model whereby $CD8^+$ MAIT cells downregulate CD8 due to continuous engagement and activation during infection. Similar rates of CD8 downregulation and DN MAIT cell death may lead to an overall contraction of the $CD8^+$ MAIT cell subset while the DN MAIT cell compartment remains stable. The suggested irreversible $CD8^+$ to DN MAIT cell transition also implies that in tissues where microbial encounter is common, such as the gut, an overall contraction of $CD8^+$ MAIT cells with time as a result of continuous engagement of resident or recruited $CD8^+$ MAIT cells could be expected. In this context, it would be interesting to investigate the dynamics of these MAIT cell subsets in such tissues during homeostasis and upon infection.

Although our own findings and those of others may support the aforementioned derivative model, direct experimental evidence of the relationships suggested are warranted, namely the irreversible transitions from $CD8^+$ to DN MAIT cells, and from DN MAIT cell to cell death. Long-term stimulation of purified $CD8^+$ and DN MAIT cell populations and evaluation of CD8 expression over time would be necessary. Assessment of CD8 intracellular expression in *ex vivo* resting DN MAIT cells at the transcript and protein level would be important for a better understanding of the true identity of DN MAIT cells. Are they not equipped at all to express CD8, or as we suggest, is CD8 re-expression somehow restricted by yet-unknown mechanisms after downregulation? Apoptosis blocking experiments following stimulation could help clarify the transition from DN MAIT cells to cell death. If our hypothesis holds true, one could in such experiments expect accumulation of DN MAIT cells, while the $CD8^+$ MAIT cell population would contract over time.

## 5.4 MAIT CELLS IN HEPATITIS DELTA

MAIT cells have been studied in a wide variety of diseases, including cancer, autoimmune diseases, as well as bacterial and viral infections. Among the latter, viral hepatitis has

received particular attention in recent years, and several reports have been published on the involvement of MAIT cells in chronic hepatitis B and C (see *Section 1.2.9.4*). Hepatitis delta, caused by HDV, is the most severe form of viral hepatitis (see *Section 1.3*). In **Paper IV**, we used precious samples from chronic HDV-infected patients to examine the levels of peripheral blood MAIT cells and their functionality in response to TCR and innate cytokine stimulations. Chronic HBV mono-infected patients and healthy controls were included as comparison groups. Furthermore, we examined liver biopsies taken from HDV-infected patients for the presence of MAIT cells, and compared the results with control biopsies.

We initially found that the levels of MAIT cells were significantly lower in HDV-infected patients than in HBV mono-infected patients and healthy controls (**paper IV**, Fig. 1A-B). In contrast, we only detected a mild decrease in MAIT cell levels in HBV mono-infected patients when compared with healthy controls (**paper IV**, Fig. 1A-B). Conventional flow cytometry analysis of the dataset revealed that residual MAIT cells in HDV-infected patients displayed an abnormal phenotype of activation and exhaustion (**paper IV**, Fig. 2A-F and Suppl. Fig. 1B). In comparison with healthy controls or HBV mono-infected patients, a cluster of MAIT cells in HDV-infected patients simultaneously expressing higher levels of CD38 and programmed death-1 receptor (PD-1), and lower levels of CD28, CD127, PLZF, Eomes, and Helios ($CD38^{hi}PD\text{-}1^{hi}CD28^{lo}CD127^{lo}PLZF^{lo}Eomes^{lo}Helios^{lo}$ cells) was detected by high-dimensional Barnes-Hut stochastic neighbor embedding (SNE) analysis (**paper IV**, Fig. 2G-H, J and Suppl. Fig. 1D, F). In contrast, differences in the phenotype of MAIT cells from HBV mono-infected patients when compared with healthy controls were only minor (**paper IV**, Fig. 2A-F, I and Suppl. Fig. 1B, E).

The mechanism(s) that drive MAIT cell loss in chronic HDV infection (or in any other viral infection where the levels of peripheral blood MAIT cells were reportedly decreased (see *Section 1.2.9.4*)) are currently unknown. However, several hypotheses have been suggested and they are discussed in *Sections 5.4.1* to *5.4.4* according to results obtained in this study and in previous studies.

### 5.4.1 Innate cytokine-mediated activation-induced MAIT cell death

Plasma cytokine measurements revealed that IL-12 and IL-18 were significantly more abundant in the plasma of HDV-infected patients than in healthy controls (**paper IV**, Fig. 4A). HDV contains a single-stranded RNA genome rich in guanine and cytosine [199, 200]. Thus, it is possible that this virus is recognized by TLR7- and TLR8- expressing cells, which in turn may produce IL-12 and IL-18 in response to viral infection, in a similar manner to what has been reported for liver monocytes upon TLR8 engagement [88]. MAIT cells may, therefore, indirectly respond to HDV through TCR-independent stimulation mediated by IL-12 and IL-18. In this context, we evaluated the *in vitro* responsiveness of residual peripheral blood MAIT cells from HDV-infected patients to the IL-12 and IL-18 cytokine combination. MAIT cells from these patients responded to a similar extent as MAIT cells from HBV mono-infected patients and healthy controls (**paper IV**, Fig. 3A-D). Given that even residual

exhausted MAIT cells responded well to innate cytokines, one can speculate that there are similarly uncompromised responses during the initial phases of infection before MAIT cells are depleted from peripheral blood. At such an early phase of infection, the MAIT cell response to innate cytokines may importantly contribute to the overall protective immune response against HDV.

Upon characterization of the surface immunoproteome of MAIT cells from healthy donors, we found that these cells were clearly distinct from other T cell populations (**paper II**, Fig. 4A-C and Suppl. Table 1). At the same time, they were highly homogeneous in their surface immune repertoire, as most of the proteins were either expressed by virtually all MAIT cells or none (**paper II**, Fig. 5A and Suppl. Table 1). Strikingly, however, MAIT cells displayed marked heterogeneity in the expression of certain NK cell-associated markers that were expressed at intermediate levels (**paper II**, Fig. 5A-C and Suppl. Table 1). Among them, we found that MAIT cells expressing CD56, CD84, and CD94 produced higher levels of IFNγ in response to IL-12 and IL-18 than their negative counterparts (**paper II**, Fig. 5D-E). The higher responsiveness of $CD56^+$ MAIT cells was associated with higher basal expression levels of Prf, IL-12R, and IL-18R, as well as of PLZF, Eomes, and T-bet (**paper II**, Fig. 6). Thus, through their higher capacity to respond to IL-12 and IL-18, $CD56^+$ MAIT cells may be particularly advantageous in the immune response against HDV. Strategic location of this MAIT cell subset at sites of inflammation, including the liver, and their relative proportion in relation to $CD56^-$ MAIT cells may help dictate the overall extent of host immune protection against HDV. The proportion of MAIT cells expressing CD56 was reported to be significantly higher in the liver than in the peripheral blood of healthy individuals [88, 89, 226]. It would thus be interesting to determine whether this is maintained throughout the course of HDV infection, and if, how and why the viral infection might compromise this cell subset over time.

Despite the intact responsiveness of residual MAIT cells to IL-12 and IL-18 stimulation, we also showed that long-term culture in the presence of these cytokines significantly increased MAIT cell death *in vitro* (**paper IV**, Fig. 4B-C). It is thus possible that continuous activation of MAIT cells by these cytokines combined with limited feedback inhibition mechanisms leaves these cells vulnerable to persistent activation, ultimately leading to their exhaustion and decline in circulation. In agreement with this hypothesis, an IL-18-driven mechanism has been suggested to similarly affect iNKT cells in the context of chronic inflammatory disease [249].

### 5.4.2 Microbe-mediated activation-induced MAIT cell death

#### *5.4.2.1 MAIT cell loss by engagement in antimicrobial responses*

It has been hypothesized that the loss of MAIT cells from peripheral blood of HIV-1-infected patients is due to continuous engagement of these cells in antimicrobial responses as a consequence of microbial translocation [92, 93, paper SI]. In fact, *in vitro* exposure to *E. coli* has been shown to induce MAIT cell apoptosis and selective loss of this cell population from

*in vitro* cultures in an MR1-dependent manner [92]. Microbial translocation has also been shown to occur in chronic HBV and HCV infections [250], and it is possible that this phenomenon also occurs during HDV infection. In this context, we studied the *in vitro* response of MAIT cells to *E. coli*, and found that MAIT cells from HDV-infected patients concomitantly failed to upregulate CD69 and CD25, to degranulate as assessed by CD107a expression, and to produce GrzB (**paper IV**, Fig. 3E, F, H and Suppl. Fig. 2C, E). Conventional flow cytometry analysis also revealed that MAIT cells from HDV-infected patients produced lower levels of IFNγ (**paper IV**, Fig. 3C-D). Overall, MAIT cell responses to TCR stimulation were impaired in HDV-infected patients, whereas MAIT cells from HBV mono-infected patients were as functional as those from healthy controls (**paper IV**, Fig. 3 and Suppl. Fig. 2C-E). The defective functionality of MAIT cells in hepatitis delta may compromise the ability of the host immune system to cope with microbial pathogens, and overall contribute to the higher severity of the disease when compared with chronic HBV infection alone.

In conclusion, in HDV infection, continuous engagement of MAIT cells in antimicrobial responses is likely to lead to their activation, exhaustion, and consequent loss from peripheral blood. Strikingly, the loss of MAIT cells specifically occurred in the $CD8^+$ MAIT cell compartment (**paper IV**, Fig. 1C and Suppl. Fig. 1A), and this finding is in agreement with its previously reported superior functionality to TCR stimulation (**paper III**, Fig. 3) (discussed in *Sections 5.2.1.2* and *5.2.1.3*). Of note, engagement and exhaustion of MAIT cells in antimicrobial responses in hepatitis delta may not only be due to TCR stimulation but also to the TCR/MR1-independent responses elicited by innate cytokines that derive from HDV-infected APCs.

#### *5.4.2.2 MAIT cell loss by CD161 downregulation following microbial stimulation*

TCR-mediated activation of MAIT cells may lead to strong downregulation of the TCR Vα7.2 segment (**paper II**, Fig. 1C) as well as of the CD8 co-receptor (**paper III**, Suppl. Fig. 2A). Similarly, downregulation of CD161 can also occur [93], and it has been hypothesized that in HIV-1 infection, the reported loss of MAIT cells from peripheral blood may be partly due to the downregulation of CD161 after activation [93]. This would consequently lead to underestimation of the frequency of MAIT cells as they become hard to distinguish from *bona fide* $CD161^-$ T cells. This hypothesis is so far controversial in the HIV/MAIT cell field. While several studies have reported an increase in the levels of $CD161^-V\alpha7.2^+$ cells during HIV-1 infection [86, 93, 172, 173, 251], and the proportion of these cells increased *in vitro* following MAIT cell exposure to *E. coli* [93], one study showed that the expanded $CD161^-V\alpha7.2^+$ cell population in HIV-1-infected patients did not stain with the MR1 5-OP-RU tetramer, therefore claiming that this population does not contain *bona fide* MAIT cells [173]. In HCV infection, the levels of $CD161^-V\alpha7.2^+$ cells in peripheral blood and liver were similar to those of healthy controls [86, 181]. In our study, we also did not detect an increase in the levels of $CD161^-V\alpha7.2^+$ cells in HDV-infected (or chronic HBV mono-infected) patients when compared with healthy controls (**paper IV**, Fig. 1A-B). Thus, it is unlikely that CD161

downregulation in MAIT cells underlies the loss of this cell population from peripheral blood in HDV-infected patients.

### 5.4.3 MAIT cell recruitment to the inflamed liver

As peripheral blood MAIT cells express CCR6 and CXCR6 [61] (see *Section 1.2.5*), one may speculate that the decline in circulating MAIT cells in HDV-infected patients may be due to their recruitment to the inflamed liver. Immunohistochemical analysis of liver biopsies from HDV-infected patients did not show signs of MAIT cell accumulation in the liver (**paper IV**, Fig. 1E), similar to previous reports on liver MAIT cells from chronic HBV-infected patients [88]. However, it is still possible that MAIT cells are recruited there and undergo apoptosis following engagement in antimicrobial responses, resulting in a lack of net MAIT cell accumulation or even a decline in the total MAIT cell pool in that organ over time. Consistent with this hypothesis, we detected a significant inverse correlation between the levels of peripheral blood MAIT cells and liver fibrosis score in hepatitis patients (**paper IV**, Fig. 1D). Moreover, in patients with chronic HCV infection [86, 180], or HBV or HVC-infected patients with end-stage liver disease [88], the levels of liver MAIT cells were significantly lower than in healthy controls [86, 88, 180]. Detailed investigation into the MAIT cells in the liver at several time points during the course of HDV infection in parallel with the analysis of clinical parameters indicative of liver inflammation would be required to better understand the numerical and functional dynamics of the hepatic MAIT cell population, and how it may be locally affected by this type of viral infection.

### 5.4.4 Conclusions on the involvement of MAIT cells in hepatitis delta

In summary, we found that the MAIT cell compartment is profoundly affected in hepatitis delta. We detected a severe decline in the levels of circulating MAIT cells in HDV-infected patients that was associated with disease severity, as assessed by the level of hepatic fibrosis. Residual circulating MAIT cells showed signs of activation and exhaustion, and were functionally impaired in response to TCR stimulation.

HBV infection alone did not significantly affect the levels or functionality of MAIT cells, a pattern consistent with a recent report [183] and with the notion that hepatitis delta presents with a more severe clinical scenario than hepatitis B alone [205, 206] (see *Section 1.3*). This also suggests that it is either HDV itself or the clinical consequences of the HBV/HDV co-infection that profoundly affect MAIT cells during hepatitis delta. Although separate hypotheses for the loss of circulating MAIT cells were presented here, it is likely that MAIT cell loss results from a dynamic combination of different mechanisms occurring in peripheral blood and in the liver. In this context, it would also be interesting to perform multivariate analysis of our dataset in order to potentially identify factors independently associated with MAIT cell loss from peripheral blood. Although MAIT cells were shown to not be prone to direct infection by HIV-1 [92], it would be interesting to evaluate whether HBV and HDV can directly infect MAIT cells. Due to their role in antimicrobial responses, it is reasonable to speculate that the severe loss of MAIT cells from peripheral blood might compromise their

ability to mount robust immune responses. This may contribute to increased sensitivity to bacterial infections in patients with severe liver disease.

MAIT cells represent a dominant fraction of the T cell compartment in the liver, and it is reasonable to believe that they constitute an important component for immunosurveillance and antimicrobial defense in this organ [159]. In support of this notion, liver MAIT cells are more activated than their blood counterparts, as assessed by their higher expression levels of CD69, CD38, and HLA-DR [89], and, together with $CD56^{bright}$ NK cells, they are the main producers of IFNγ following TLR8 stimulation of liver mononuclear cells [88]. However, due to the same fact that liver MAIT cells are highly activated and able to rapidly secrete pro-inflammatory cytokines, one may wonder about their contribution to the pathogenesis of the disease. Mechanistic studies, clinical correlation analyses, and *in vivo* models may help understand the role of MAIT cells in hepatitis delta [159]. Both the innate and adaptive immune systems are weakened during chronic HDV infection (see *Section 1.3*), and understanding their respective roles as well as those of the innate-like T cell populations, such as MAIT cells, in the protection or pathogenesis of hepatitis delta may help devise efficient therapeutic strategies.

# 6 CONCLUDING REMARKS AND FUTURE DIRECTIONS

When the work that constitutes this thesis started, research on human MAIT cells had just begun, their antigens had not yet been identified, and there were no published studies on their involvement in viral diseases. In a relatively short period of time, extensive research in the field has greatly advanced our knowledge of the immunobiology of MAIT cells and their role in the immune system.

Specifically in this thesis, we established MAIT cell-based experimental platforms that can be used to study several functions of these cells and adapted to answer a wide variety of research questions. We showed that MAIT cell responses to distinct riboflavin biosynthesis-competent microbes depend on the microbial dose and differ in the type and amount of cytokines produced. We demonstrated functional compartmentalization of the MAIT cell population, as the TCR β chain composition and CD8 expression, as well as the expression of NK cell-associated receptors, influence their capacity to respond to TCR and innate cytokine stimulation, respectively. In an attempt to understand the relationship between $CD8^+$ and DN MAIT cells, we showed that $CD8^+$ MAIT cells are functionally superior to DN MAIT cells and that the latter may derive from the former *in vivo*. Finally, we studied MAIT cells in hepatitis delta and showed that they are severely depleted from the peripheral blood of HDV-infected patients, with the residual MAIT cells being activated, exhausted, and functionally impaired in response to TCR stimulation.

Although specific future experiments have been suggested throughout the discussion of the results of this thesis (*Section 5*), the panel of questions about MAIT cells that remains to be clarified is immense. We have come to know a lot about MAIT cell effector functions, but much remains to be understood about how they are regulated and modulated *in vitro* and *in vivo*. Strikingly, the precise function of CD161 has been scarcely studied in the MAIT cell field, and the few experiments aimed at investigating its modulatory role have led to controversial results. Furthermore, what role does NKG2D have on MAIT cells? Its expression was detected in peripheral blood MAIT cells, but whether it is also expressed in tissue MAIT cells remains to be determined, as does the effect thereof. On the other hand, the production of pro-inflammatory mediators, such as IL-17, appears to be tightly regulated, and investigations into how this regulation is achieved and which factors are involved are of utmost importance, especially if these cells are to be used in immunotherapeutic approaches in the future.

The involvement of MAIT cells in several diseases is clear. It is particularly striking that MAIT cells are depleted from the circulation in most of the diseases where they have been investigated, but their recruitment to affected tissues was not always assessed, and even when so, not always observed. Whether their sometimes-reported dysfunctionality is a consequence or a cause of the disease has not been determined, and, therefore, their exact role as protective, pathogenic, or modulatory also remains elusive. It is challenging to investigate

these types of questions in humans, but the use of animal models and the study of sophisticated cohorts of patients may, in the future, shed light on these questions.

Given the abundance of MAIT cells at sites of microbial encounter such as the gut, one can speculate that failure in their capacity to discriminate between commensal and pathogenic microbes would have drastic consequences to the human body. Thus, full understanding of the mechanisms and factors that allow such discrimination is required, and will greatly advance our knowledge of MAIT cell immunobiology. One may also wonder how the microbiota influences this cell population postnatally. Strikingly, could the microbiota be involved in shaping the abundance of MAIT cells, and therefore contribute to the high inter-donor variability in circulating MAIT cell levels? The finding that CD1d-deficient mice have more MAIT cells suggests that competition with other innate T cell populations for the same niche may occur [63]. If that is the case, the complexity of this question goes beyond MAIT cells alone to include the investigation of factors affecting the abundance of other T cell populations as well.

The co-existence of T cells able to recognize antigens of different nature - peptides, lipids, and vitamin metabolites - arms the human body with a highly specialized system that can perform immunosurveillance at many different fronts and defend against a wide array of pathogens. MAIT cells occupy a distinct niche within the global T cell compartment: they are abundant in several tissues and recognize a remarkably different set of antigens. In a way, one can say that it is surprising that their identification has taken so long. Interestingly enough, though, researchers were already studying part of this cell population when focusing on circulating CD161-expressing $CD8^+$ T cells in a wide variety of contexts. Given the novelty of these cells and the immense progress that has been made in a short period of time, one can only look forward in anticipation to the coming years of MAIT cell research.

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*"A goal is a dream with a deadline."*
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