MAIT cell diversity, function and impact on dendritic cells

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Abbreviations

0-9	
5-A-RU	5-amino-6-D-ribitulaminouracil
5-OE-RU	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
6-FP	6-formylpterin
7AAD	7-aminoactinomycin D
A	
APC	antigen-presenting cell
B	
BSA	bovine serum albumin
C	
CBA	cytometric bead array
CCL	chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
cDC	conventional dendritic cell
cDNA	complementary DNA
CDR	complementarity determining region
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CTV	cell Trace Violet
CXCR	C-X-C chemokine receptors
D	
d	day
DC	dendritic cell
DN	double negative

DNA	deoxyribonucleic acid
DP	double positive
E	
eGFP	enhanced Green Fluorescent Protein
F	
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FOXP3	forkhead box P3
G	
GITR	glucocorticoid-Induced TNF (Tumor Necrosis Factor) Receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
H	
h	hour
I	
ICOS	inducible T-cell costimulator
IEL	intra-epithelial lymphocytes
IFNγ	interferon gamma
IL	interleukin
i.t.	intratracheal
i.v.	intravenous
L	
LN	lymph node
LPS	lipopolysaccharide
M	
MACS	magnetic activated cell sorting
MAIT	mucosal associated invariant T cell
medLN	mediastinal lymph node
MeG	methylglyoxal
MFI	mean fluorescence intensity

	MHC	major histocompatibility complex
	MR1	MHC related protein 1
	mRNA	messenger RNA
N		
	NKT	natural killer T cell
O		
	OT-I	
	OVA	ovalbumin
P		
	PAMPs	pathogen associated molecular pattern
	PBMCs	peripheral blood mononuclear cells
	PBS	phosphate buffered saline
	PCR	polymerase chain reaction
	PLZF	promyelocytic leukaemia zinc finger
	PMA	phorbol myristate acetate
	PRR	pattern recognition receptor
Q		
	qRT-PCR	quantitative Real-Time PCR
R		
	RNA	ribonucleic acid
	RoRγT	retinoic acid-related orphan receptor gamma
S		
	SI	small intestine
T		
	T-bet	T-box transcription factor
	TCR	T-cell receptor
	T_H	T helper cell
	TLR	Toll-like receptor
	TNFα	tumor necrosis factor alpha
	TRAC	T cell receptor alpha constant
	TRAJ	T cell receptor alpha joining
	TRAV	T cell receptor alpha variable

TRBC	T cell receptor beta constant
TRBJ	T cell receptor beta joining
TRBV	T cell receptor beta variable
T_{reg}	regulatory T cell

α

αGalCer	alpha-galactosylceramide

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Abstract

T cells represent an important component of the immune system. Whilst early studies were largely focused on the role of conventional $CD8^+$ and $CD4^+$ T cells that recognize peptide-antigens in association with MCH molecules, more recently, T cells that recognize other types of antigens have been described. Mucosal associated invariant T (MAIT) cells are such a cell population and belong to the broad family known as 'unconventional' T cells, due to their non-peptidic antigen recognition characteristics. MAIT cells are defined by their recognition of microbial vitamin B2 metabolites presented by MHC related protein 1 (MR1). Upon antigen recognition they immediately display effector functions, like secreting cytokines and expression of cytotoxic proteins. Whilst the majority of MAIT cell studies have focused on the role of MAIT cells to bacterial infections, however their function within the immune system and interaction with other immune cells is still unknown. This thesis focuses on the role that MAIT cell activation has on other immune cells like dendritic cells (DCs) and other T cells. Furthermore, the full potential of MR1-recognition by other T cell subsets was also examined, revealing that MR1-reactive T cells may extend beyond what is currently describe as MAIT cells.

The first chapter of this thesis investigates the role of MAIT cell activation on DCs in an *in vivo* mouse model. MAIT cells were activated by intratracheal injection of the activating MAIT cell antigen 5-amino-6-D-ribitulaminouracil/ methylglyoxal (5-A-RU/MeG). This activation of MAIT cells led to migration of DCs from the lung to the mediastinal lymph node (medLN) as well as DC maturation in an MR1-dependent manner. Furthermore, production of the chemokines CCL17 and CCL22 was induced by MAIT cell activation, which suggests that MAIT cells are able to modulate the immune system far more than previously thought. The possible role of MAIT cell induced DC maturation on initiation of a $CD8^+$ T cell response is analyzed within the second result chapter. No enhanced antigen-specific $CD8^+$ T cell response to the model antigen ovalbumin (OVA) was observed by additional MAIT cell activation.

Besides MAIT cells, recently more MR1-reactive T cells were identified. By using antigen-loaded MR1 tetramers, a population of $FOXP3^+$ $T\text{-}bet^+$ T cells was identified in human thymus that can bind to MR1 tetramers. In the third chapter this $FOXP3^+$ $T\text{-}bet^+$ T cell population was further characterized by analysis of their phenotype as well as their TCR usage. The results in this chapter will serve as a basis for further investigation of the diversity of MR1-recognition within the T cell pool.

In conclusion, this thesis reveals a new role of MAIT cells that may be used to manipulate their functions to treat different diseases like autoimmune diseases or cancer. Moreover, the knowledge of MR1-reactive T cell diversity is extended including a potential regulatory role of MR1-reactive T cells and MAIT cells. In summary, this thesis extends the current knowledge of MAIT cell biology.

Declaration

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This is to certify that,

- **(i)** the thesis comprises only my original work towards the PhD except where indicated in the preface,
- **(ii)** due acknowledgement has been made in the text to all other material used
- **(iii)** the thesis is fewer than the maximum word limit in length, exclusive of tables, maps, bibliographies and appendices.

Marie-Sophie Philipp

Preface

My contribution to the experiments within each chapter was as follows:

Chapter 3: 98 %

Chapter 4: 100 %

Chapter 5: 90 %

I acknowledge the important contributions of others to the experiments presented herein:

Chapter 3: Katherine Gourley (repeated CBA experiment with higher dilution of cell culture supernatant (Fig. 3.13))

Chapter 5: Dr. Garth Cameron (provided Fig. 5.1), Dr. Hui-Fern Koay (processed one thymus), Rebecca Seneviratna (isolated PBMCs from human blood used in Fig 5.15)

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List of publications

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Kelly, J., Minoda, Y., Meredith, T., Cameron, G., **Philipp, M**., Pellicci, D.G., Corbett, A.J., Kurts, C., Gray, D.H., Godfrey, D.I., Kannourakis, G. and Berzins, S.P. (2019) 'Chronically Stimulated Human MAIT Cells Are Unexpectedly Potent IL-13 Producers'. *Immunology & Cell Biology*. DOI: 10.1111/imcb.12281.

Enders, M.*, Franken, L.*, **Philipp, M.-S.***, Kessler, N.*, Baumgart, A.-K., Eichler, M., Wiertz, E.J.H., Garbi, N. and Kurts, C. (2019) 'Splenic Red Pulp Macrophages Cross-Prime Early Effector CTL That Provide Rapid Defense against Viral Infections'. *The Journal of Immunology*, p. ji1900021. DOI: 10.4049/jimmunol.1900021.

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1. Chapter 1: Introduction

1.1. The immune system

The immune system defends the host against infections and cancer. A variety of cells and molecules cooperate for this purpose. Generally, the immune system can be divided into two different arms: the innate and adaptive immune system.

The innate immune system is the first line of defense and acts through soluble factors and innate cells, such as macrophages, granulocytes and natural killer (NK) cells. These cells act fast after recognizing pathogens through pathogen-associated molecular patterns (PAMPs). In contrast, the adaptive immune system mounts specific immune responses by lymphocytes (Murphy *et al.*, 2012). These are distinguished into B and T lymphocytes (Miller, 1961; Cooper *et al.*, 1966) that can recognize a great variety of different antigens by their B cell receptors (BCRs) or T cell receptors (TCRs). During the immune response some of these cells can differentiate into memory cells, which are responsible for long-lasting immunity. If a second exposure of antigen occurs, memory cells can rapidly differentiate into effector cells leading to a fast and specific secondary immune response (Murphy *et al.*, 2012).

1.2. T lymphocytes

T lymphocytes, which are also called T cells, develop from multipotential lymphoid precursors that migrate from the bone marrow to the thymus (Wu *et al.*, 1991; Kondo *et al.*, 1997). These are initially double negative (DN: CD4-CD8-) and pass through four different stages, which can be distinguished by their expression of CD25 and CD44. DN1 are $CD25^-$ $CD44^+$, DN2 are $CD25^+$ $CD44^+$, DN3 are $CD25^+$ $CD44^-$ and DN4 are

$CD25^-$ $CD44^-$ (Godfrey *et al.*, 1993). Following their development in stage 3, a pre-αTCR is expressed that pairs with a already rearranged TCRβ chain (Saint-Ruf *et al.*, 1994). This pre-TCR interacts with different proteins (CD3/TCRζ) on the cell surface that in turn leads to signal transduction (van Oers, 1995). Such signaling is required for the development and further maturation of T cells (Van Oers *et al.*, 1996). After pre-TCR signaling, CD4 and CD8 are upregulated on DN cells so that they progress to the double positive (DP, $CD4^+$ $CD8^+$) stage. RAG genes are expressed and TCRα recombination occurs (Koch and Radtke, 2011). Those DP thymocytes can recognize peptide: major histocompatibility complex (MHC) complexes presented on cortical thymic epithelial cells (cTECs) via their T cell receptor (TCR). Following a low-avidity interaction, the cells receive survival signals and can differentiate into $CD4^+$ or $CD8^+$ single positive (SP) cells, depending on their affinity to either MHC class I or MHC class II (Takaba and Takayanagi, 2017). This process is also termed positive selection. In contrast, cells undergo cell death if a high-avidity interaction between TCR and MHC molecule occurs. This mechanism is also termed negative selection and helps to delete self-reactive T cells. DP thymocytes undergo programmed cell death if a TCR-MHC interaction is absent. This process is called death by neglect (Takahama, 2006).

After development, T cells recirculate through the body via the blood stream and the lymphoid organs and lymphatic vessels. T cells that have not encountered antigen yet are referred to as naïve T cells that need activation to perform their specialized functions. For activation, a T cell needs to encounter its antigenic peptide, which is presented by an antigen-presenting cell (APC) on MHC class I or class II molecules. The peptide:MHC complexes are recognized by the TCR (Murphy *et al.*, 2012) and during ligation a signal is transferred to CD3 that builds a complex with the TCR (Wucherpfennig *et al.*, 2010). The cytosolic part of CD3 induces an intracellular signal leading to activation and proliferation (Samelson, 2002). Besides CD3, CD4 or CD8 are also

associated with the TCR on the cell surface. CD4 or CD8 molecules are responsible for stabilization of the TCR-MHC complex because they bind to the different MHC molecules. CD4 binds to MHC class II molecules, while CD8 binds to MHC class I molecules. So $CD8^+$ T cells can only recognize peptides presented by MHC class I molecules, while $CD4^+$ T cells can recognize peptides loaded onto MHC class II molecules (Doyle and Strominger, 1987; Norment *et al.*, 1988).

After the T cell has recognized its specific antigen, it starts to proliferate and develops into effector T cells. $CD8^+$ T cells differentiate into cytotoxic T cells (CTLs) after antigen recognition. Cytotoxic T cells are able to kill infected target cells or tumor cells that present foreign antigens on their cell surface via MHC class I. Interferon γ (IFNγ) and tumor-necrosis factor α (TNFα) are expressed by CTLs to kill a target cell. Furthermore, CTLs start to produce molecules like granzymes, perforin and Fas ligand, which kill target cells (Andersen *et al.*, 2006).

In contrast, $CD4^+$ T cells differentiate into different T helper cells (T_H) upon antigen recognition of peptides presented by MHC class II molecules. The different T_H cells are termed T_H1, T_H2 and T_H17 and their differentiation depends on the cytokine milieu present during activation (Kaech *et al.*, 2002; Luckheeram *et al.*, 2012).

T_H1 cells develop in the presence of IL-12 (Hsieh *et al.*, 1993). The IL-12 signal leads to expression of T-bet, which in turn enhances production of IFNγ. IFNγ is one of the signature cytokines produced by T_H1 cells and these cells are important or defense against intracellular bacteria (Luckheeram *et al.*, 2012).

T_H2 cells develop from $CD4^+$ T cells that encounter IL-4 in the environment. STAT6 is induced by IL-4, which in turn leads to expression of GATA-binding factor 3 (GATA3) (Zhu *et al.*, 2001). They are mainly responsible for defense against parasites and produce IL-4 and IL-13 (Kara *et al.*, 2014; Bao and Reinhardt, 2015).

Besides T_H1 and T_H2 cells, $CD4^+$ T cells can also differentiate into T_H17 cells. Such differentiation requires IL-6 plus transforming growth factor-β

(TGFβ) (Veldhoen *et al.*, 2006). IL-6 induces STAT3, which in turn leads to expression of the transcription factor retinoid-related orphan receptor-γt (RoRγT) (Yang *et al.*, 2007). The major feature of T_H17 cells is the production of IL-17 that is directly regulated by RoRγT (Ivanov *et al.*, 2006). T_H17 cell are important for the defense against extracellular infections, but have also been implicated in autoimmune conditions (Maddur *et al.*, 2012; Kara *et al.*, 2014).

In addition to these three main T_H cell subsets, T_H9, T_H22 and T follicular helper cells (T_{FH}) have been distinguished. These cell subsets play a role in the defense of extracellular infections. Furthermore, T_{FH} are associated with the development of antigen-specific B cells (Kara *et al.*, 2014).

After first antigen recognition, T cells differentiate into primary effector cells. After clearance of infection or tumor cells, the differentiated cells can develop into memory cells that provide long lasting immunity and can be activated rapidly upon a second recognition of antigen (Pennock *et al.*, 2013).

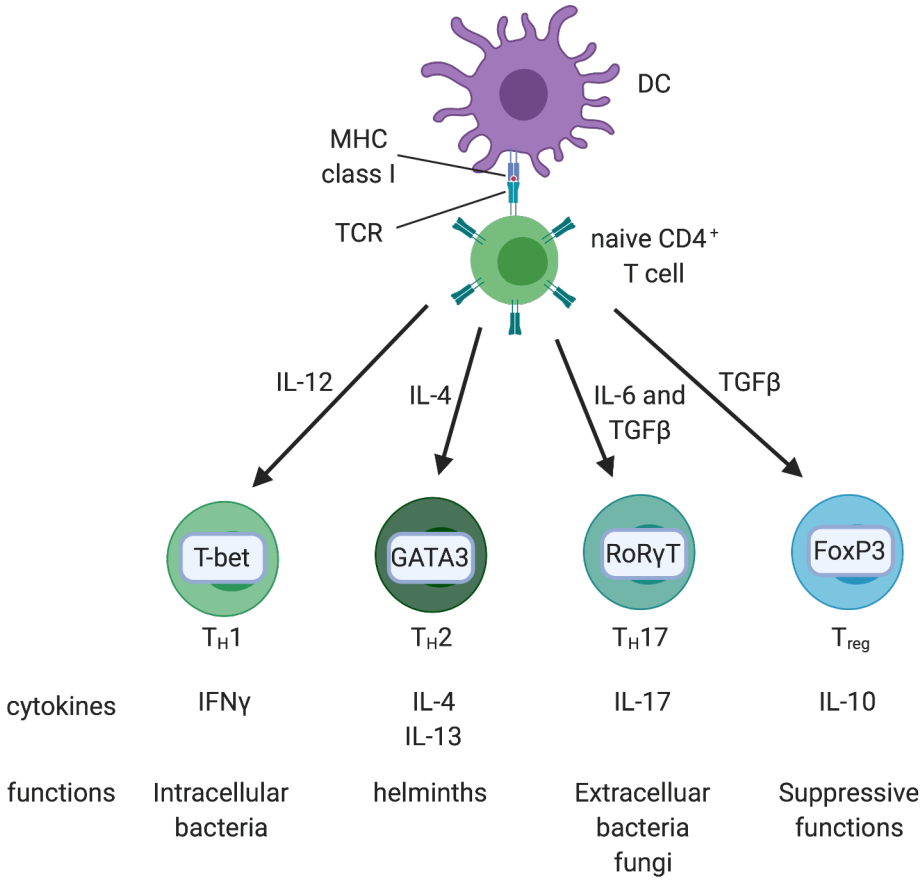


Figure 1. 1 – Differentiation of naïve $CD4^+$ T cells. Differentiation of naïve $CD4^+$ T cells is dependent on the cytokine environment. The different cytokines induce expression of different transcription factors that lead to specific cytokine production. The different T_H cell subsets are shown here with the different transcription factors as well as the cytokine production after differentiation and their function in host defense.

1.3. Regulatory T cells

Regulatory T cells (T_{regs}) are identified as cells that express the transcription factor forkhead box P3 (FoxP3) and the surface molecule CD25. FoxP3 is the master regulator of T_{regs} (Fontenot *et al.*, 2005). T_{regs} are reported to suppress different immune cells and immune responses (Schmidt *et al.*, 2012). By direct interaction of T_{regs} with APCs, downregulation of co-stimulatory molecules occurs, which in turn

suppresses activation of conventional T cells. Furthermore, T_{regs} produce suppressive cytokines like IL-10 and TGFβ to perform their suppressive function (Ouyang *et al.*, 2011; Schmidt *et al.*, 2012; Luckheeram *et al.*, 2012).

T_{regs} can be induced in the periphery by $CD4^+$ T differentiation in the presence of TGFβ that induces the expression of forkhead box protein 3 (FoxP3) (Chen *et al.*, 2003) and are called peripherally derived T_{regs} (pT_{regs}).

Moreover, some T_{regs} also develop within the thymus and are called thymic derived T_{regs} (tT_{reg}). In humans, those thymic T_{regs} can already be found in fetuses (Cupedo *et al.*, 2005), while the numbers of tT_{regs} are stable from fetus to infant thymuses (Darrasse-Jèze *et al.*, 2005). Mainly CD4SP cells, as well as CD8SP and DP express the T_{reg} markers FoxP3 and CD25 and give rise to T_{regs} in human thymuses (Tuovinen *et al.*, 2008). In the DP stage, T_{regs} express high levels of CD3 as well as CD27, showing a mature phenotype (Nunes-Cabaço *et al.*, 2011). Besides FoxP3 and CD25, T_{regs} in thymuses express markers that are associated with T_{reg} function. So DP $FoxP3^+$ $CD25^+$ cells express CTLA-4, GITR and CD39 (Cupedo *et al.*, 2005; Nunes-Cabaço *et al.*, 2011), while the T_{reg} marker ICOS is expressed by CD4SP $FoxP3^+$ $CD25^+$ cells (Ito *et al.*, 2008).

1.4. Unconventional T cells

Besides $CD4^+$ and $CD8^+$ T cells that are also named ‘conventional’ T cells and that recognize peptide MHC complexes, other T cells are present that recognize non-polymorphic antigen-presenting molecules, which include MR1-restricted T cells, CD1-restricted T cells, MHC class Ib-reactive T cells and γδ T cells. Their unique feature is the recognition of non-peptide antigens, like lipids, small-molecule metabolites and modified peptides. They are referred to as unconventional T cells and are not donor-restricted because all the antigen-presenting molecules

are ubiquitously expressed. Besides unconventional T cells, they are also called innate-like T cells and can respond rapidly to antigen stimulation.

Natural killer T cells (NKT) cells are restricted by the CD1d molecule, which is loaded with glycolipids like α-galactosylceramide (α-GalCer). NKT cells represent about 1-3 % of T cells in tissues of mice and rapidly produce cytokines and become effector cells. They can activate DCs by upregulation of CD40L and their cytokine production (Godfrey *et al.*, 2015).

1.4.1. Mucosal associated invariant T cells (MAIT cells)

Mucosal associated invariant T cells (MAIT cells) are T cells that were first discovered by Porcelli and colleagues in 1993. They were described as a DN ($CD8^-CD4^-$) T cell population that expresses the α-chain Vα7.2 (TRAV1-2) Jα33 (Porcelli *et al.*, 1993). This α-chain is mainly paired with Vβ6 and Vβ20.1 (Held *et al.*, 2015). Later, MAIT cells were described in mice as well. In mice, the Vα19 (TRAV1) Jα33 α-chain is used in their TCRs (Tilloy *et al.*, 1999) and it is paired mainly with Vβ8 and Vβ6 (Rahimpour *et al.*, 2015). Since MAIT cells are enriched in the gut mucosa, they were named mucosal associated invariant T cells (MAIT cells) (Treiner *et al.*, 2003), but now it is known that they are located within various tissues (Dusseaux *et al.*, 2011; Rahimpour *et al.*, 2015). In human blood around 1-10% of all T cells are MAIT cells and for example in the liver they make up to 45% of all T cells (Le Bourhis *et al.*, 2010; Dusseaux *et al.*, 2011). Interestingly, in mice their frequency is very low. For example, only up to 0.1% of all T cells are MAIT cells in the blood of mice, while the highest frequency was found in lungs (3% of all T cells) (Rahimpour *et al.*, 2015).

MAIT cells recognize, in contrast to conventional T cells, microbial metabolites of the riboflavin (vitamin B2) biosynthesis pathway (Kjer-Nielsen *et al.*, 2012). It was shown that bacteria lacking specific enzymes of the riboflavin pathway are not able to activate MAIT cells

(Corbett *et al.*, 2014). Furthermore, it was shown that a precursor metabolite 5-amino-6-D-ribitylaminouracil (5-A-RU) could react with small molecules like glyoxal or methylglyoxal (MeG) in a non-enzymatic reaction. The reaction of 5-A-RU and MeG generates the molecule 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), which was shown to activate MAIT cells and bind to the MHC related protein 1 (MR1) (Corbett *et al.*, 2014). Besides those metabolites, folic acid sources, for example 6-formyl-pterin (6-FP), were identified to bind MR1 as well. It was shown that 6-FP can upregulate MR1 expression on the cell surface, but it is not able to activate MAIT cells like 5-OP-RU (Kjer-Nielsen *et al.*, 2012).

MR1 is the antigen-presenting protein for MAIT cells. It is highly conserved between species, with 90 % sequence identity between humans and mice (Riegert *et al.*, 1998). MR1 is expressed ubiquitously in various cell types and organs (Huang *et al.*, 2008). It is stored within the endoplasmic reticulum (ER), where it can be loaded with antigen, leading to a conformational change and binding to β2m. After binding of antigen, the MR1 complex traffics to the cell surface, where it is internalized within an hour and is recycled or degraded (McWilliam *et al.*, 2016).

Upon activation MAIT cells mainly produce the cytokines IFNγ, TNFα an IL-17A (Dusseaux *et al.*, 2011; Rahimpour *et al.*, 2015). They also rapidly express granzyme B and perforin after activation and are able to kill infected cells (Kurioka *et al.*, 2015). Besides sensing bacterial infections due to microbial antigen recognition, MAIT cells are able to sense viral infections. Wilgenburg *et al.* showed that MAIT cells are able to sense viral infection in a cytokine-dependent manner. The cytokines IL-12 and IL-18 are mainly responsible for MAIT cell activation (Wilgenburg *et al.*, 2016).

MAIT cells possess an activated memory tissue-targeted phenotype. In humans they express CD26, CD27, CD28, CD127 and CD45RO, but are negative for CD62L and CD25. They also show expression of the

chemokine receptors CCR2, CCR5, CCR6, CXCR4 and CXCR6 in PBMCs. In contrast, they do not express CCR7 and CXCR3 (Dusseaux *et al.*, 2011; Brozova *et al.*, 2016).

Murine MAIT cells are characterized by expression of CD44. Besides this marker they also express CD103, CD127, CXCR6 and IL-18R depending on the tissue. Additionally, they are negative for the markers CD69, CD62L and CCR9. The transcription factors expressed by MAIT cells are PLZF as well as RoRγT and T-bet. Two different subsets of MAIT cells were identified depending on the transcription factors RoRγT and T-bet. RoRγT$^+$ MAIT cells are IL-17A producers, while T-bet$^+$ MAIT cells mainly produce IFNγ (Rahimpour *et al.*, 2015).

1.4.2. MAIT cell development

The development of MAIT cells takes place in the thymus (Tilloy *et al.*, 1999; Martin *et al.*, 2009). It is thought that T cells randomly rearrange their TCR and if the TCR can interact with MR1 on DP thymocytes, the cells are selected into MAIT cell linage (Seach *et al.*, 2013). Using antigen-loaded MR1 tetramers, three different developmental stages were identified in the thymus. In mice, these stages differ in the expression of CD24 and CD44, while stage 1 is CD24$^+$CD44$^-$, stage 2 is CD24$^-$CD44$^-$ and stage 3 is CD24$^-$CD44$^+$. During transition from stage 2 to stage 3 MR1 as well as microbial antigens and promyelocytic leukaemia zinc finger (PLZF) are important. In stage 3 MAIT cells express classical MAIT cell markers like IL-18R and resemble mature MAIT cells. Within the thymus all three different stages are present, but with low numbers of stage 3 MAIT cells. In contrast, in the periphery only stage 3 is present. MAIT cell development in human thymus is similar to the development found in mice. Also here three stages were identified, but with the use of different surface molecules. Stage 1 is identified as CD27$^-$ CD161$^-$ cells, stage 2 as CD27$^+$ CD161$^-$ and stage 3 as CD27$^+$ CD161$^+$ cells. Similar to murine MAIT cell development, stage 3 MAIT cells express classical MAIT cell markers, like IL-18R and

PLZF. Also here the transcription factor PLZF is important for the transition of stage 2 to stage 3 MAIT cells. Again, all stages can be found within thymus, while only stage 3 MAIT cells are present in the periphery. In the periphery MAIT cells undergo further maturation and expansion (Koay *et al.*, 2016).

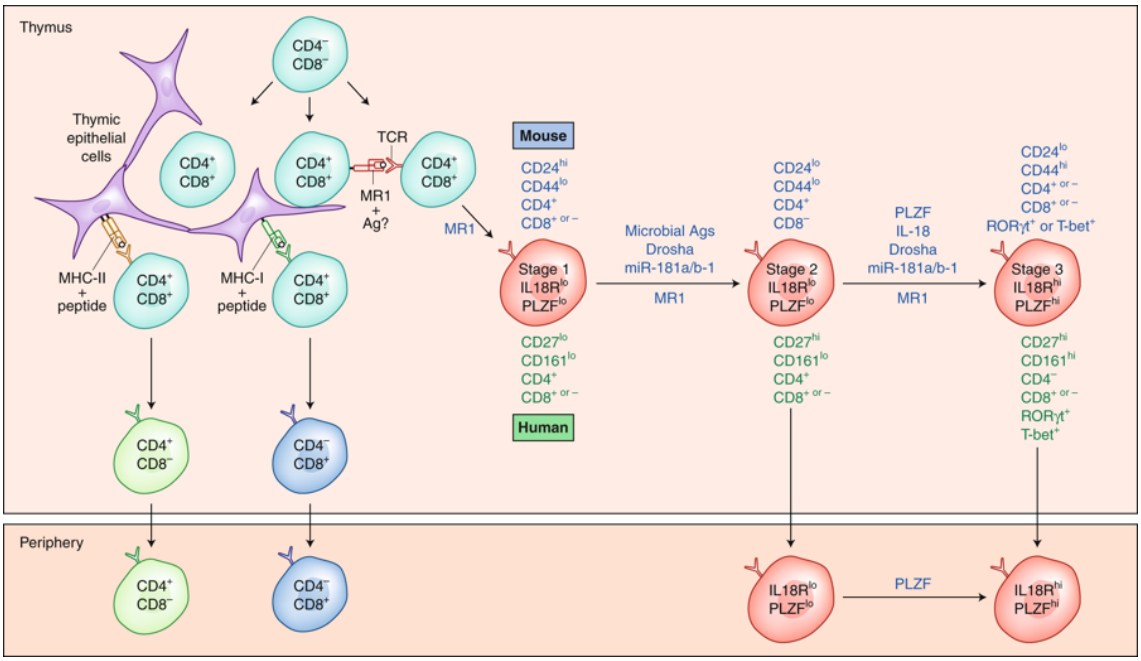


Figure 1. 2 – Development of MAIT cells in mice and human. On the left side the development of conventional T cells is shown. DP thymocytes interact with DP thymocytes via MR1, leading to development of MAIT cells. Development from stage 1 to stage 3 is shown with expression of the different markers in mice and humans. Reprinted from Godfrey *et al.*, 2019 with permission**.**

1.4.3. Effect of MAIT cells on other immune cells

MAIT cells are able to kill infected cells via direct interaction, but also interactions with other immune cells have been described. It was shown that MAIT cells promote early differentiation of monocytes into monocyte-derived DCs during *Francisella tularensis* infection. This differentiation was driven by MAIT cell-dependent GM-CSF production. GM-CSF in turn led to recruitment of CD4+ T cells after *F. tularensis* infection (Meierovics and Cowley, 2016). Furthermore, it was shown

that human MAIT cells are able to induce DC maturation *in vitro*. Upon activation, MAIT cells upregulate CD40L that leads to maturation of DCs and production of IL-12 by DCs (Salio *et al.*, 2017).

Moreover, MAIT cells were able to provide cognate B cell help *in vitro*. By producing soluble factors, MAIT cells promote differentiation of memory B cells into plasmaplasts with increased antibody production (Bennett *et al.*, 2017).

Also MR1- and cytokine dependent transactivation of NK cells was shown in whole blood after MAIT cell activation (Salio *et al.*, 2017).

Since most of those studies were performed *in vitro,* it still needs to be investigated if those interactions can take place *in vivo.* So a lot of open questions remain about MAIT cells and their immune regulatory function within the immune system.

1.4.4. Diversity of MR1-resticted T cells

Besides MAIT cells, a variety of other MR1-restricted cells was found. Different studies suggested the existence of MR1-resticted populations with diverse TCR repertoire and antigen specificities. Cells that can bind to MR1 tetramers but do not express the typical MAIT cell α-chain Vα7.2 (TRAV1-2) were found in human PBMCs (Gherardin *et al.*, 2016) and were subdivided into two different classes of MR1-reactive T cells. First, cells with a non MAIT-like phenotype. They do not express typical MAIT cell markers like CD161 and IL-18R (CD218a) and their TCR is highly diverse with no conservation in CDR3α and β junctional motif or length. Second, cells with a MAIT-like phenotype that express typical MAIT cell markers like CD161 and IL-18R. Those MAIT-like cells can be further divided into two different subsets. One subset with a diverse TCR gene usage, while the other subset that expresses a TCR consisting of TRAV36 TRAJ34/37 TRBV28/25-1 TRBJ2-5. Those cells also have a CDR3α and CDR3β of a invariant length of 11 or 14 amino acids while the CDR3α sequence is highly germline encoded and the CDR3β sequence had a semi-invariant motif (Koay *et al.*, 2019). In another

study one clone was discovered that expressed the T cell receptor alpha variable 12-2 (TRAV12-2) and reacted to *Streptococcus pyogenes*, a bacterial pathogen that do not express the enzymes of the riboflavin biosynthesis pathway. This suggest that besides the known antigens of MAIT cells more antigens can be recognized by MR1-reactive T cells (Meermeier *et al.*, 2016). Furthermore, TRAV1-2$^-$ cells were described that react against MR1-overexpressing cells without antigen stimulation (Lepore *et al.*, 2017).

In mice, MAIT cells were identified that uses another TCR as the previous described TCR with the α-chain TRAV1 TRAJ33. In Jα33 knockout mice a few MAIT cells remained that uses mainly TRAV1 and TRAV6 that are paired with various J segments. So basically two different MR1-reactive T cells can be found in Jα33 knockout mice. First TRAV1+ cells and second TRAV1- cells. Besides various J genes they have a bias towards TRBV13 usage (Koay *et al.*, 2019).

1.5. Dendritic cells

Dendritic cells were first described by Steinman in 1973 (Steinman *et al.*, 1975). They are specialized antigen-presenting cells that process antigens and present them to T cells (Nussenzweig *et al.*, 1980).

DCs can be classified into two main subsets, conventional DC1 (cDC1) and conventional DC2 (cDC2), which are characterized by CD103 (cDC1) or CD11b (cDC2) expression in non-lymphoid tissues, respectively. cDC1s are dependent on Batf3 and IRF8 and express these transcription factors after development, while cDC2s are dependent on IRF4 (Tamura *et al.*, 2005; Hildner *et al.*, 2008). cDC1s are known to play a role in CD8$^+$ T cell priming by a mechanism called cross-presentation. In contrast, cDC2s are able to present antigens to CD4$^+$ T cells. This leads to induction of T_H cells or T_{regs} (Den Haan *et al.*, 2000a; Pooley *et al.*, 2001; Eisenbarth, 2019).

DCs develop from a common myeloid progenitor (CMP) into a macrophage-dendritic cell progenitor (MDP) (Fogg *et al.*, 2006). During further development, they differentiate into either a common monocyte progenitor, which give rise to monocytes (Hettinger *et al.*, 2013), or a common dendritic cell progenitor (CDP), that develops into pre-pDCs or pre-DCs (Naik *et al.*, 2007). Those pre-DCs travel through the blood to the spleen and other tissues, where they develop into conventional DCs (Liu and Nussenzweig, 2010).

DCs are located in different tissues. There, they capture antigens and process them. After upregulation of co-stimulatory molecules, they start to migrate to the lymph nodes or the spleen (Banchereau *et al.*, 2000), where they can transfer the antigen to lymph node resident cells or present the antigen directly to T cells that leads to induction of T cell activation (Allan *et al.*, 2006). Depending on the tissue cDC1 or cDC2 can migrate to the lymph node. For example in the lung both subsets are able to migrate to the mediastinal lymph node (Plantinga *et al.*, 2013; Krishnaswamy *et al.*, 2017).

During infection, injury or vaccination, DCs start to mature. During this process they upregulate MHC class II surface expression as well as co-stimulatory molecules like CD80, CD86 and CD40. They start to migrate in a CCR7-dependent manner and produce cytokines to promote T cell differentiation. Depending of the stimuli, they can produce different cytokines to induce different T cell differentiation (Dalod *et al.*, 2014).

The activation of DCs is driven by pattern-recognition receptors (PRRs) that detect molecular patterns that are conserved by invading microorganisms and called pathogen-associated molecular patterns (PAMPs) or damaged cells with patterns called damage-associated molecular patterns (DAMPs). Four classes of PRR were identified so far. Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) are those PRRs (Walsh *et al.*, 2013; Amarante-Mendes *et al.*, 2018). TLRs are the most studied PRRs so far. In humans 10 members

were described, while in mice 13 TLRs exist. They are transmembrane receptors, consisting of an extracellular leucine-rich repeat (LRR) and an intracellular Toll/IL-1 receptor (TIR) domain. TLRs are located at the cell surface or intracellular and recognize different PAMPs (McGettrick and O'Neill, 2010). TLR4 for example recognize bacterial lipopolysaccharide (LPS) (Hoshino *et al.*, 1999; Lu *et al.*, 2008). TLR5 is expressed on the surface of epithelia cells as well as monocytes and immature DCs and recognize bacterial flagellin (Hayashi *et al.*, 2001; Yang and Yan, 2017). Besides proteins, also RNA or DNA can be sensed by TLRs. Double-stranded RNA (dsRNA) is recognized by intracellular TLR3 (Alexopoulou *et al.*, 2001). In contrast, single-stranded RNA (ssRNA) is recognized by TLR7 (Diebold *et al.*, 2004), while bacterial DNA is recognized by TLR9. Especially unmethylated CpG motifs are recognized by this receptor. Interestingly, most CpG motifs are methylated in mammals, while these motifs are unmethylated in bacteria, leading to the differentiation between host and pathogen (Hemmi *et al.*, 2000). TLR stimulation with LPS or CpG can be use to induce to maturation of DCs (Sparwasser *et al.*, 1998; Michelsen *et al.*, 2001).

1.6. Cross-priming

Dendritic cells can present peptides on MHC molecules. Intracellular antigens are presented on MHC class I molecules, which are recognized by $CD8^+$ T cells, while extracellular antigens are taken up by DCs and are loaded onto MHC II molecules that are recognized by $CD4^+$ T cells (Moore *et al.*, 1988; Yewdell *et al.*, 1988). Besides this, exogenous antigens can also be presented on MHC class I molecules and lead to $CD8^+$ T cell response (Bevan, 1976; Moore *et al.*, 1988; Yewdell *et al.*, 1988; Carbone and Bevan, 1990). This phenomenon is also called cross-presentation (Kurts *et al.*, 1996). Cross-presentation is the basis for a mechanism called cross-priming. Cross-priming was first described

1976 (Bevan, 1976). It describes the mechanism where a $CD8^+$ T cell response is initiated with an antigen that is cross-presented by an APC. Dendritic cells are the major cell type that is able to cross-present antigens *in vivo*. Especially $CD103^+$/ $CD8^+$ DCs are specialized in cross-priming (Den Haan *et al.*, 2000a; del Rio *et al.*, 2007). Upon antigen encounter in the peripheral tissue, specific DCs take up the antigens and transport them to the lymph nodes. Then DCs transfer the antigen to specialized DCs that cross-prime $CD8^+$ T cells (Allan *et al.*, 2006).

Antigen recognition by $CD8^+$ T cells is not the only signal needed to initiate a proper $CD8^+$ T cell response. Therefore, antigen recognition is referred to as signal 1, while co-stimulatory molecules on DCs are postulated as signal 2 (Bretscher and Cohn, 1970). Those co-stimulatory molecules, like CD80 and CD86, are upregulated, if a DC encounters antigen together with pathogen-associated molecular pattern (PAMP) for example toll-like-receptors (TLR) and can enhance cross-priming (Maurer *et al.*, 2002). Besides the postulated two signals, it was shown that a third signal is needed for efficient cross-priming. This third signal is provided by cytokines that are produced by DCs, leading to proliferation and differentiation of $CD8^+$ T cells (Curtsinger *et al.*, 1999).

Besides stimulation with PAMPs, another factor is need, which is provided by CD4+ T cells (Husmann and Bevan, 1988). $CD4^+$ T cells provide this signal by CD40L-CD40 ligation (Bennett *et al.*, 1998; Schoenberger *et al.*, 1998). Ligation of CD40 also leads to upregulation of co-stimulatory molecules and production of cytokines like IL-12 (Yang and Wilson, 1996; Bennett *et al.*, 1998; Schulz *et al.*, 2000). Furthermore, inhibitory molecules like PD-L1 are downregulated by interaction of $CD4^+$ T cells with DCs leading to activation of $CD8^+$ T cells (Keir *et al.*, 2007). This process of DC activation by $CD4^+$ T cell help is also called DC licensing (Kurts *et al.*, 2010). $CD8^+$ T cells that are activated without $CD4^+$ T cell help are called ‘helpless’ $CD8^+$ T cells. They do not have effector functions and have a short life-span (Janssen *et al.*, 2005).

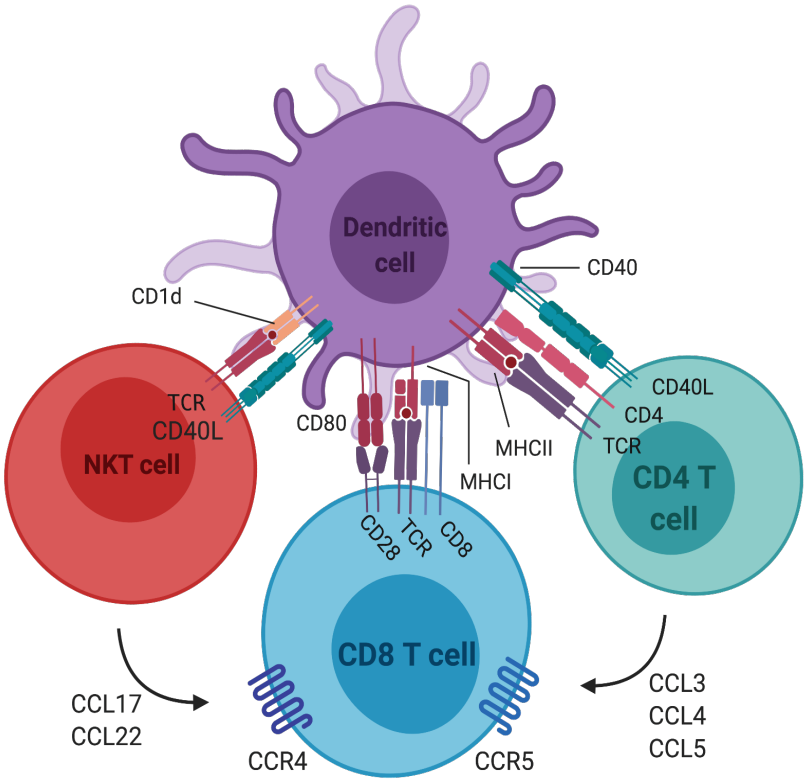


Figure 1. 3 - Mechanisms of cross-priming. $CD4^+$ T cells or NKT cells are able to provide help to a dendritic cell via CD40-CD40L interaction as well as TCR recognition. The DC upregulates co-stimulatory molecules like CD80 and cross-presents peptide to $CD8^+$ T cells, which gets primed and receive survival signals. Furthermore, recruitment of $CD8^+$ T cells with the appropriate chemokine receptors (CCR) is mediated by chemokines expressed by NKT cells, $CD4^+$ T cells as well as DCs. Figure is adapted from Kurts, Robinson and Knolle, 2010 with permission.

Besides $CD4^+$ T cells, also NKT cells are able to enhance $CD8^+$ T cell response (Nishimura *et al.*, 2000; Ian F Hermans *et al.*, 2003). NKT cells provide help to the DC by interacting through CD1d loaded with αGalCer presented by the DC. NKT cells are able upregulate on co-stimulatory molecules on DCs (Fujii *et al.*, 2003; Fujii *et al.*, 2007). Additionally CD40L is expressed on NKT cells upon recognition of αGalCer-loaded CD1d leading to CD40-dependent licensing of DCs (Fujii *et al.*, 2004). Furthermore, NKT cells induce IL-12 production by DCs (Tomura *et al.*,

1999). Cross-priming mediated by NKT cells is also called alternative cross-priming (Semmling *et al.*, 2010), while $CD4^+$ T cell mediated cross-priming is referred to as classical cross-priming. By depletion of $CD4^+$ T cells it was shown that the alternative cross-priming is completely independent of $CD4^+$ T cell help (Semmling *et al.*, 2010).
Besides the cell-cell contacts and the described signals leading to $CD8^+$ T cell priming, an additional signal was identified, which is provided by chemokines. Chemokines are produced by DCs as well as T cells and play an important role during cross-priming. They are important for recruitment of $CD8^+$ T cells and therefore referred to as signal 0 (Bousso and Albert, 2010). Classical licensed DCs produce CCL3 and CCL4, which lead to recruitment of $CCR5^+$ $CD8^+$ T cells (Castellino *et al.*, 2006). In contrast, alternative licensed DCs produce CCL17 leading to recruitment of $CCR4^+$ $CD8^+$ T cells (Semmling *et al.*, 2010).

1.7. Chemokines

Chemokines are cytokines with chemotactic features. Around 50 chemokines are known in humans and mice. The corresponding receptors consist of G-protein-coupled chemokine receptors atypical chemokine receptors. Around 20 signaling receptors are known while 5 non-signaling receptors were identified. Chemokines and the receptors control migration and the positions of immune cells. Furthermore they are required for immune responses (Griffith *et al.*, 2014). For example it was shown that DCs upregulate CCR7 upon stimulation and can migrate to the lymphatics and the LN in a CCR7-dependent manner. Chemokines that bind to CCR7 are CCL19 and CCL21. Lymphatic vessels produce CCL21 in non lymphoid organs, which lead to migration of $CCR7^+$ DCs upon stimulation (Griffith *et al.*, 2014). Furthermore, naïve T cells express CCR7 leading to homing of naïve T cells in the LN. Upon activation, T cells downregulate CCR7 but start to upregulate CCR2, CCR3, CCR4, CCR5, CCR6, CCR8 and CXCR5 leading to

recirculation of T cells (Bachmann *et al.*, 2006). Moreover, chemokines and chemokine receptors play a role in tissue homing of T cells. Memory T cells express CCR4 for trafficking to the lung and the skin. In contrast to CCR4, CCR9 is known to be important for gut homing (Griffith *et al.*, 2014).

1.8. Thesis aims

MAIT cells are an unconventional T cell population that is relatively new in immunology. The function of MAIT cells was mostly described in infection models, consistent with their ability to recognize microbial metabolites. With the discovery of an activating antigen and the development of specific tetramers for identification of MAIT cells, new opportunities have opened to analyze MAIT cells and their functions. As only few studies have reported roles of MAIT cells in immunity, I here aimed at analyzing the factors that regulate their function, especially their effect to modulate DCs and their function as well as the ability to enhance cross-priming. Furthermore, it is aimed to further study the diversity of MR1 recognition. Therefore, 3 different aims are investigated in this thesis:

1. **Chapter 3: Do MAIT cell activation affect DC maturation and function?**
   Interaction of MAIT cells with the immune system and its cells remain poorly defined. Therefore the effect of MAIT cell activation on DCs was investigated in *in vivo* studies.

2. **Chapter 4: Do MAIT cells enhance $CD8^+$ T cell priming?**
   Whether MAIT cells can enhance $CD8^+$ T cell priming like NKT cells is an important question. Thus, in this chapter, vaccination based strategies were used to answer this question.

3. **Chapter 5: What is the phenotype of MR1-reactive T cells in human thymus?**

   Besides MAIT cells other MR1-reactive T cells were described. This chapter deals with the characterization of newly identified MR1-reactive T cells in human thymus.

2. Chapter 2: Material and Methods

2.1. Material

2.1.1. Mice

Table 2. 1 - Mouse strains used in this thesis

Line	Background	Description
C57BL/6J		C57BL/6J mice were purchased from Charles River, Janvier or bred in-house at the University of Bonn, House of Experimental Therapy (HET) or at the Department of Microbiology and Immunology Biological Research Facility (BRF).
CCL17eGFP	C57BL/6N	These mice express an eGFP knock-in construct under the control of the CCL17 promotor.
$MR1^{-/-}$	C57BL/6J	This mouse strain lack the protein MR1 that is important for the development and activation of MAIT cells. Since MAIT cells are restricted to MR1, these mice lack MAIT cells. Dr. Jan-Eric Turner (Universitätsklinikum Eppendorf, Hamburg, Germany) kindly provided the mice.
OT-I	C57BL/6J	This mouse strain expresses a transgenic T cell receptor that recognizes the OVA peptide SIINFEKL ($OVA_{257-264}$) in H-$2K^b$ molecules. Almost exclusively all T cells are expressing the transgenic TCR.
CD45.1	C57BL/6J	This mouse strain expressed the allelic variant of the pan leukocyte marker CD45 known as CD45.1.

2.1.2. Human samples

Human thymus samples were obtained from the Royal Children's Hospital, Victoria, Australia. The samples were processed and analyzed directly after surgical removal. All experiments were performed according to the University of Melbourne Medicine and Dentistry Human Ethics Committee (reference number 1035100).

Healthy adult peripheral blood samples were obtained from the Australian Red Cross Blood Service. From this blood samples PBMCs were isolated. In this study only frozen PBMCs previously isolated from blood were used.

2.1.3. Antibodies

2.1.3.1. Mouse

Table 2. 2 – Anti-mouse antibodies used in this thesis

Antigen	**Clone**	**Conjugate**	**Company**
B220	RA3-6B2	PE	Biolegend
		BV786	
CCR4	2G12	PE	Biolegend
CCR6	29-2L17	BV421	Biolegend
CD103	2E7	PEDazzle594	Biolegend
		APC	
		PerCPCy5.5	
		BV421	
CD11b	M1/7	BV711	Biolegend
		BUV395	
CD11c	HL3	BUV737	BD
CD19	6D5	APCCy7	Biolegend
	1D3	BV510	BD
CD25	PC61.5	APC	Biolegend
		PE	
CD28	37,51	Purified NA/LE	BD Bioscience

CD3ε	145-2C11	Purified NA/LE	BD Bioscience
CD4	GK1.5	BV421	Biolegend
		PECy7	
		BV510	
CD40	3/23	PECy7	Biolegend
CD44	IM7	BV421	Biolegend
		FITC	
		PECy7	
		APCCy7	
		biotin	
CD45	30-F11	APCCy7	Biolegend
CD45.2	104	BV711	Biolegend
		PerCPCy5.5	
CD69	H1.2F3	PerCPCy5.5	Biolegend
CD8	53-6.7	AF700	Biolegend
		APC	
		BV510	
		PerCPCy5.5	
CD80	16-10A1	PerCPCy5.5	Biolegend
CD86	GL1	APC	Biolegend
GM-CSF	MP1-22E9	FITC	eBioscience
IFNγ	XMG1.2	PECy7	BD Bioscience
IL-10	JES5‐16E3	AF647	Biolegend
IL-13	eBio13A	PE	eBioscience
IL-17	eBio17B7	PerCPCy5.5	eBioscience
	TC11-18H10		BD Bioscience
MHCII	M5/114.15.2	FITC	Biolegend
	M5/114.15.2	AF700	Biolegend
NK1.1	PK136	PECy7	eBioscience
PD-L1	10F.9G2	PB	Biolegend
SiglecF	E50-2440	BV605	Biolegend

TCRβ	H57-597	BV711	Biolegend
		BV421	
		APC	
TCRγδ	GL3	PerCPCy5.5	Biolegend
Vβ6	RR4-7	APC	Biolegend
Vβ8	KJ16-133.18	AF647	Biolegend

2.1.3.2. Human antibodies

Table 2. 3 – Anti-human antibodies used in this thesis

Antigen	**Clone**	**Conjugate**	**Company**
AhR	T49-550	PE	BD Bioscience
CCR5	2D7/CCR5	BV605	BD Bioscience
CCR6	IIA9	BUV496	BD Bioscience
CCR7	G043H7	BV786	Biolegend
CCR9	BBC3M4	eFluor660	eBioscience
CD103	Ber-ACT8	BV785	Biolegend
CD127	eBioRDR5	PECy7	eBioscience
	A019D5	PerCPCy5.5	Biolegend
CD14	MΦP9	APCCy7	BD Bioscience
		BUV805	
CD183 (CXCR3)	G025H7	PECy7	Biolegend
		PerCPCy5.5	
CD19	SJ25C1	APCCy7	BD Bioscience
CD194 (CCR4)	L29IH4	APC	Biolegend
CD212	2.4E6	APC	BD Bioscience
CD218a (IL-18Ra)	H44	PE	Biolegend
		APC	eBioscience
CD25	BC96	APC	eBioscience
		BV650	Biolegend
		APCCy7	BD Bioscience

CD278 (ICOS)	ISA-3	eFluor450	eBioscience
CD28	CD28.2	Purified NA/LE	BD Bioscience
CD3	UCHT1	AF700	BD Bioscience
		BV421	BD Bioscience
		BUV395	BD Bioscience
		Purified NA/LE	BD Bioscience
CD31	WM59	BV421	Biolegend
CD357 (GITR)	108-17	APC/Fire750	Biolegend
CD4	RPA-T4	AF700	BD Bioscience
CD45RA	HI100	PerCPCy5.5	BD Bioscience
CD45RO	UCHL1	BV421	Biolegend
CD8α	SK1	AlexaFluor488 APC	Biolegend
CD8β	SIDI8BEE	PECy7	Biolegend
CTLA4	BNI3	BV421	Biolegend
FoxP3	259D	AF647	Biolegend
	206D	BV421	Biolegend
GM-CSF	BVD2-2ICII	PE/Dazzle594	Biolegend
IFNγ	4S.B3	BV650	BD Bioscience
IL-13	JES10-5A2	PE	BD Bioscience
IL-17A	eBio64DEC17	APC	eBioscience
T-bet (anti-human/mouse)	eBio4B10	PECy7	eBioscience
TNF	MAb11	PECy7	BD Bioscience
Vα7.2	3C10	BV711	Biolegend
		FITC	Biolegend
		PE	Biolegend

2.1.4. Cytometric bead array

For human cytokine samples the Flex sets for IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, GM-CSF, IFNγ and TNFα (BD Bioscience) were used. For murine samples the Flex sets for IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17A, GM-CSF, IFNγ and TNFα (BD Bioscience) were used.

2.1.5. Primer

2.1.5.1. Multiplex PCR

2.1.5.1.1. External TRAV primer sequences

Table 2. 4 - External TRAV primer sequences

Primer	**Sequence 5'→3'**
hTRAC_Ext	GAC CAG CTT GAC ATC ACA G
hTRAV1_Ext	AAC TGC ACG TAC CAG ACA TC
hTRAV2_Ext	GAT GTG CAC CAA GAC TCC
hTRAV3_Ext	AAG ATC AGG TCA ACG TTG C
hTRAV4_Ext	CTC CAT GGA CTC ATA TGA AGG
hTRAV5_Ext	CTT TTC CTG AGT GTC CGA G
hTRAV6_Ext	CAC CCT GAC CTG CAA CTA TAC
hTRAV7_Ext	AGC TGC ACG TAC TCT GTC AG
hTRAV8-1_Ext	CTC ACT GGA GTT GGG ATG
hTRAV8-2_8-4_Ext	GCC ACC CTG GTT AAA GG
hTRAV8-3_Ext	CAC TGT CTC TGA AGG AGC C
hTRAV8-6_Ext	GAG CTG AGG TGC AAC TAC TC
hTRAV8-7_Ext	CTA ACA GAG GCC ACC CAG
hTRAV9-1_9-2_Ext	TGG TAT GTC CAA TAT CCT GG
hTRAV10_Ext	CAA GTG GAG CAG AGT CCT C
hTRAV12-1_12-2_12-3_Ext	CAR TGT TCC AGA GGG AGC

hTRAV13-1_Ext	CAT CCT TCA ACC CTG AGT G
hTRAV13-2_Ext	CAG CGC CTC AGA CTA CTT C
hTRAV14_Ext	AAG ATA ACT CAA ACC CAA CCA G
hTRAV16_Ext	AGT GGA GCT GAA GTG CAA C
hTRAV17_Ext	GGA GAA GAG GAT CCT CAG G
hTRAV18_Ext	TCC AGT ATC TAA ACA AAG AGC C
hTRAV19_Ext	AGG TAA CTC AAG CGC AGA C
hTRAV20_Ext	CAC AGT CAG CGG TTT AAG AG
hTRAV21_Ext	TTC CTG CAG CTC TGA GTG
hTRAV22_Ext	GTC CTC CAG ACC TGA TTC TC
hTRAV23_Ext	TGC TTA TGA GAA CAC TGC G
hTRAV24_Ext	CTC AGT CAC TGC ATG TTC AG
hTRAV25_Ext	GGA CTT CAC CAC GTA CTG C
hTRAV26-1_Ext	GCA AAC CTG CCT TGT AAT C
hTRAV26-2_Ext	AGC CAA ATT CAA TGG AGA G
hTRAV27_Ext	TCA GTT TCT AAG CAT CCA AGA G
hTRAV29_Ext	GCA AGT TAA GCA AAA TTC ACC
hTRAV30_Ext	CAA CAA CCA GTG CAG AGT C
hTRAV34_Ext	AGA ACT GGA GCA GAG TCC TC
hTRAV35_Ext	GGT CAA CAG CTG AAT CAG AG
hTRAV36_Ext	GAA GAC AAG GTG GTA CAA AGC
hTRAV38-1_38-2_Ext	GCA CAT ATG ACA CCA GTG AG
hTRAV39_Ext	CTG TTC CTG AGC ATG CAG
hTRAV40_Ext	GCA TCT GTG ACT ATG AAC TGC
hTRAV41_Ext	AAT GAA GTG GAG CAG AGT CC

2.1.5.1.2. External TRBV primer sequences

Table 2. 5 - External TRBV primer sequences

Primer	**Sequence 5'→3'**
hTRBC_Ext	TAG AAC TGG ACT TGA CAG CG
hTRBV2_Ext	TCG ATG ATC AAT TCT CAG TTG

hTRBV3-1_Ext	CAA AAT ACC TGG TCA CAC AG
hTRBV4-1-3_Ext	TCG CTT CTC ACC TGA ATG
hTRBV5-1_5-3-4_Ext	GAT TCT CAG GKC KCC AGT TC
hTRBV5-5-8_Ext	GTA CCA ACA GGY CCT GGG T
hTRBV6-1-3_6-5-9_Ext	ACT CAG ACC CCA AAA TTC C
hTRBV6-4_Ext	ACT GGC AAA GGA GAA GTC C
hTRBV7-1-3_Ext	TRT GAT CCA ATT TCA GGT CA
hTRBV7-4_7-6-9_Ext	GSW TCT YTG CAG ARA GGC C
hTRBV9_Ex	GAT CAC AGC AAC TGG ACA G
hTRBV10-1-3_Ext	TGT WCT GGT ATC GAC AAG ACC
hTRBV11-1-3_Ext	CGA TTT TCT GCA GAG ACG C
hTRBV12-3-5_Ext	ARG TGA CAG ARA TGG GAC AA
hTRBV13_Ext	AGC GAT AAA GGA AGC ATC C
hTRBV14_Ext	CCA ACA ATC GAT TCT TAG CTG
hTRBV15_Ext	AGT GAC CCT GAG TTG TTC TC
hTRBV16_Ext	GTC TTT GAT GAA ACA GGT ATG C
hTRBV17_Ext	CAG ACC CCC AGA CAC AAG
hTRBV18_Ext	CAT AGA TGA GTC AGG AAT GCC
hTRBV19_Ext	AGT TGT GAA CAG AAT TTG AAC C
hTRBV20-1_Ext	AAG TTT CTC ATC AAC CAT GC
hTRBV23-1_Ext	GCG ATT CTC ATC TCA ATG C
hTRBV24-1_Ext	CCT ACG GTT GAT CTA TTA CTC C
hTRBV25-1_Ext	ACT ACA CCT CAT CCA CTA TTC C
hTRBV27_28_Ext	TGG TAT CGA CAA GAC CCA G
hTRBV29-1_Ext	TTC TGG TAC CGT CAG CAA C
hTRBV30_Ext	TCC AGC TGC TCT TCT ACT CC

2.1.5.1.3. Internal TRAV primer sequences

Table 2. 6 - Internal TRAV primer sequences

Primer	**Sequence 5'→3'**
hTRAC_Int	TGT TGC TCT TGA AGT CCA TAG

hTRAV1_Int	GCA CCC ACA TTT CTK TCT TAC
hTRAV2_Int	CAC TCT GTG TCC AAT GCT TAC
hTRAV3_Int	ATG CAC CTA TTC AGT CTC TGG
hTRAV4_Int	ATT ATA TCA CGT GGT ACC AAC AG
hTRAV5_Int	TAC ACA GAC AGC TCC TCC AC
hTRAV6_Int	TGG TAC CGA CAA GAT CCA G
hTRAV7_Int	ACA ATT TGC AGT GGT ACA GG
hTRAV8-1_Int	GTC AAC ACC TTC AGC TTC TC
hTRAV8-2_8-4_Int	AGA GTG AAA CCT CCT TCC AC
hTRAV8-3_Int	TTT GAG GCT GAA TTT AAG AGG
hTRAV8-6_Int	AAC CAA GGA CTC CAG CTT C
hTRAV8-7_Int	ATC AGA GGT TTT GAG GCT G
hTRAV9-1_9-2_Int	GAA ACC ACT TCT TTC CAC TTG
hTRAV10_Int	GAA AGA ACT GCA CTC TTC AAT G
hTRAV12-1_12-2_12-3_Int	AAG ATG GAA GGT TTA CAG CAC
hTRAV13-1_Int	TCA GAC AGT GCC TCA AAC TAC
hTRAV13-2_Int	CAG TGA AAC ATC TCT CTC TGC
hTRAV14_Int	AGG CTG TGA CTC TGG ACT G
hTRAV16_Int	GTC CAG TAC TCC AGA CAA CG
hTRAV17_Int	CCA CCA TGA ACT GCA GTT AC
hTRAV18_Int	TGA CAG TTC CTT CCA CCT G
hTRAV19_Int	TGT GAC CTT GGA CTG TGT G
hTRAV20_Int	TCT GGT ATA GGC AAG ATC CTG
hTRAV21_Int	AAC TTG GTT CTC AAC TGC AG
hTRAV22_Int	CTG ACT CTG TGA ACA ATT TGC
hTRAV23_Int	TGC ATT ATT GAT AGC CAT ACG
hTRAV24_Int	TGC CTT ACA CTG GTA CAG ATG
hTRAV25_Int	TAT AAG CAA AGG CCT GGT G
hTRAV26-1_Int	CGA CAG ATT CAC TCC CAG
hTRAV26-2_Int	TTC ACT TGC CTT GTA ACC AC
hTRAV27_Int	CTC ACT GTG TAC TGC AAC TCC

hTRAV29_Int	CTG CTG AAG GTC CTA CAT TC
hTRAV30_Int	AGA AGC ATG GTG AAG CAC
hTRAV34_Int	ATC TCA CCA TAA ACT GCA CG
hTRAV35_Int	ACC TGG CTA TGG TAC AAG C
hTRAV36_Int	ATC TCT GGT TGT CCA CGA G
hTRAV38-1_38-2_Int	CAG CAG GCA GAT GAT TCT C
hTRAV39_Int	TCA ACC ACT TCA GAC AGA CTG
hTRAV40_Int	GGA GGC GGA AAT ATT AAA GAC
hTRAV41_Int	TTG TTT ATG CTG AGC TCA GG

2.1.5.1.4. Internal TRBV primer sequences

Table 2. 7 – Internal TRBV primer sequences

Primer	**Sequence 5'→3'**
hTRBC_Int	TTC TGA TGG CTC AAA CAC AG
hTRBV2_Int	TTC ACT CTG AAG ATC CGG TC
hTRBV3-1_Int	AAT CTT CAC ATC AAT TCC CTG
hTRBV4-1-3_Int	CCT GCA GCC AGA AGA CTC
hTRBV5-1_5-3-4_Int	CTT GGA GCT GGR SGA CTC
hTRBV5-5-8_Int	TCT GAG CTG AAT GTG AAC G
hTRBV6-1-3_6-5-9_Int	GTG TRC CCA GGA TAT GAA CC
hTRBV6-4_Int	TGG TTA TAG TGT CTC CAG AGC
hTRBV7-1-3_Int	TCY ACT CTG AMG WTC CAG CG
hTRBV7-4_7-6-9_Int	TGR MGA TYC AGC GCA CA
hTRBV9_Int	GTA CCA ACA GAG CCT GGA C
hTRBV10-1-3_Int	TCC YCC TCA CTC TGG AGT C
hTRBV11-1-3_Int	GAC TCC ACT CTC AAG ATC CA
hTRBV12-3-5_Int	CYA CTC TGA RGA TCC AGC C
hTRBV13_Int	CAT TCT GAA CTG AAC ATG AGC
hTRBV14_Int	ATT CTA CTC TGA AGG TGC AGC
hTRBV15_Int	ATA ACT TCC AAT CCA GGA GG
hTRBV16_Int	GAA AGA TTT TCA GCT AAG TGC C

hTRBV17_Int	TGT TCA CTG GTA CCG ACA G
hTRBV18_Int	CGA TTT TCT GCT GAA TTT CC
hTRBV19_Int	TTC CTC TCA CTG TGA CAT CG
hTRBV20-1_Int	ACT CTG ACA GTG ACC AGT GC
hTRBV23-1_Int	GCA ATC CTG TCC TCA GAA C
hTRBV24-1_Int	GAT GGA TAC AGT GTC TCT CGA
hTRBV25-1_Int	CAG AGA AGG GAG ATC TTT CC
hTRBV27_28_Int	TTC YCC CTG ATY CTG GAG TC
hTRBV29-1_Int	TCT GAC TGT GAG CAA CAT GAG
hTRBV30_Int	AGA ATC TCT CAG CCT CCA GAC

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

Table 2. 8 – Primer for qRT-PCR used in this thesis

Primer	**Sequence**
mCCL17 1	TGGTATAAGACCTCAGTGGAGTGTTC
mCCL17 2	GCTTGCCCTGGACAGTCAGA
mCCL22 1	GAGTTCTTCTGGACCTCAAATCC
mCCL22 2	TCTCGGTTCTTGACGGTTATCA
18S rRNA 1	GTAACCCGTTGAACCCCATT
18S rRNA 2	CCATCCAATCGGTAGTAGCG
GAPDH_for	GGGAAGCCCATCACCATCTT
GAPDH_rev	GCCTCACCCCATTTGATGTT

2.1.6. Buffer

Table 2. 9 – Buffer including composition used in this thesis

Buffer	**Composition**
FACS buffer	PBS + 2 % FCS or

	PBS + 0.1 % BSA or 0.2 % FCS + 0.1 % NaN_3
MACS buffer	PBS + 0.5 % FCS or BSA + 2mM EDTA
Digestion medium	RPMI medium + collagenase (1 mg/ml) + DNase (100 µg/ml)
Saponin buffer	PBS + 0.5 % Saponin
PFA	PBS + 2 % PFA
Red blood cell lysis buffer	146 mM NH4Cl + 10 mM NaHCO3 + 2 mM EDTA

2.1.7. Media

Table 2. 10 – Media used in this thesis

Medium	**Composition**
Cell culture medium	RPMI-1640 + 10 %FCS + 2 mM L-Glutamine + 0.1 mM Non-Essential Amino acids + 15 mM HEPES + 100 U/ml Penicillin + 100 U/ml Streptamycin + 1 mM Sodium Pyruvate + 50 mM 2-Mercaptoethanol RPMI-1640 + 10 % FCS + 1 % L-Glutamine + 1 % Pen/Strep + 0.5 mM β-Mercaptoethanol
LB (Luria broth) medium	Media Preparation Unit, University of Melbourne

2.1.8. Equipment

Table 2. 11 – Equipment used in this thesis

Equipment	**Company**
autoclave	Belimed
BSC	HeraSafe, Heraeus

	SafemateEco, EuroClone
	BH2000, Clyde-Apac
	Safemate 1.8 Vision, EuroClone
Cell counting chamber	Neubauer, Brand
Cell Sorter	FACSAria III, BD Bioscience
	FACSAria Fusion, BD Bioscience
	MoFlo Astrios, Beckman Coulter
Centrifuge	5810 R, Eppendorf
Centrifuge	5430 R, Eppendorf
Flow cytometer	Fortessa, BD Bioscience
	Canto II, BD Bioscience
Freezer (-20 °C)	MEDline, Liebherr
Freezer (-80 °C)	Heraeus
Fridge	Liebherr
Glas bottles	Schott Duran bottles, Duran
Heating block	Ratek
Ice machine	Icematic, Scotsman®, Frimont Bettolinc
Incubator	Heracell VIOS 160i, Thermo Scientific
	HeraCell 240, Heraeus
Light Cycler	Light Cycler 480, Roche
MACS cell seperator	QuadroMACS, Miltentyi Biotec
Measuring cylinders	Schott
Microscope	CKX31 and CX23, Olympus
	Leica DMIL, Leica Microsystems
Mini centrifuge	Micro one, Tomy
Nanodrop	Nanodrop Lite, Thermo Scientific
PCR machine	Vapo protect, Eppendorf
	PCR Mastercycler, Eppendorf
pH meter	Hanna instruments
Pipette boy	Pipet Filler, Thermo Scientific

pipettes	2.5, 10, 20, 200, 1000 µl Research plus, Eppendorf
Vortex mixer	Ratek VWR

2.1.9. Chemicals

Table 2. 12 – Chemicals used in this thesis

Chemical	Company
2-log DNA ladder	New England BioLabs
2-Mercaptoethanol (50mM)	Thermo Fisher Scientific
2x PCR master mix	Promega
5-amino-6-(D-ribitylamino)uracil (5-A-RU)	Provided by Prof. Dirk Menche, University Bonn
Agarose	Scientifix
Ampicillin	Sigma Aldrich
Big Dye Buffer	Applied Biosystems
Big Dye v3.1	Applied Biosystems
Bovine serum albumin	Sigma
BSA Fraction V	PAN-Biotech
CD11c MicroBeads UltraPure, mouse	Miltenyi
CD45R (B220) MicroBeads, mouse	Miltenyi
CD8a+ T Cell Isolation Kit, mouse	Miltenyi
cDNA reverse transcriptase	Thermo Fisher
Collagenase	Sigma Aldrich
CpG	TIB Molbiol
CytoFix/ CytoPerm	BD Bioscience
Dimethylsulfoxid (DMSO)	Carl Roth
DNase	Sigma Aldrich
Dynabeads Mouse T-Actiator CD3/CD28	Gibco, Thermo Fisher
Ethanol	Chem-Supply

ExoSAP-IT	Affymetrix
FcBlock	CSL Behring
	BD Bioscience
FcBlock	BD
Foetal Calf Serum (FCS)	FCS
FoxP3/Transcription Factor Staining Buffer Set	Affymetrix eBioscience
FuGENE HD reagent	Promega
Glutamax	Gibco
GolgiPlug	BD
Ionomycin	Sigma-Aldrich
Isoflurane	Piranal Healthcare
L-Glutamine (200mM)	Sigma Aldrich
Luria Agar (LA) plates + 100 mg/ml ampicillin	Media Preparation Unit, University of Melbourne
Luria broth	Media Preparation Unit, University of Melbourne
Methylglyoxal	Sigma Aldrich
Molecular Biology Agarose	Biorad
N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES)	Gibco
Natrium acid	Sigma Aldrich
NEBuffer	New England BioLabs
Non-essential amino acids	Gibco
PBS	Media Preparation Unit, University of Melbourne
PBS tablets	Gibco
Phytohemagglutinin (PHA)	Sigma
PMA	Sigma-Aldrich
rhIL-2	Peprotech
rhIL-7	Peprotech
RPMI-1640	Gibco

Saponin	Sigma Alrich
Sodium pyruvate	Gibco
Sterile water for Irrigation	Baxter
Streptavidin-BV421	Biolegend
Streptavidin-PE	BD Bioscience
Streptavidin-PE (Molecular Probes)	Life Technologies
Streptomycin and penicillin	Gibco or Sigma Aldrich
SuperScript VILO cDNA Synthesis Kit and Master Mix	Invitrogen
SYBER Safe DNA gel stain	Invitrogen
SYBR Green PCR MasterMix	Thermo Fisher
T4 ligase and buffer	Promega
ZymoPURE Plasmid Miniprep System Kit	Zymogen

2.1.10. Consumables

Table 2. 13 – Consumables used in this thesis

Consumable	**Company**
MACS Column	Miltenyi
96-Well plate	VWR
48 well plate	VWR
12 well plate	VWR
50 ml falcons	Greiner
15 ml falcons	Greiner
10 ml falcons	Sarstedt
FACS tubes	Sarstedt
10 µl tips	TipOne, StarLab
200 µl tips	TipOne, StarLab
1 ml tips	Greiner Bio-One
5 ml stripette	Sarstedt
10 ml stripette	Sarstedt

25 ml stripette	Sarstedt
1.5 ml Eppendorf tubes	Sarstedt
0.5 ml tubes	Sarstedt
5 ml tubes	Eppendorf
PCR tube	Biozym Scientific
Syringe (1ml)	Labomedic
Injection needles	Labomedic
Filter (100 µm)	Labomedic
Filter tips (10 µl, 200 µl, 1000 µl)	Nerbe-plus
384 well PCR plate	Roche, Eppendorf

2.1.11. Dyes

Table 2. 14 – Dyes used in this thesis

Dye	**Company**
7-aminoactinomycin D (7AAD)	Sigma Aldrich
CellTrace Violet	Molecular probes, Life Technologies
CFSE	Thermo Fisher Scientific
Fixable Viability Dye eFluor 506	eBioscience
Fixable Viability Dye eFluor 780	eBioscience
Hoechst 33342	Molecular Probes, Life Technologies
PKH26	Sigma-Aldrich

2.1.12. Programs

Table 2. 15 – Programs used in this thesis

Program	**Company**
Adobe Illustrator CS6	Adobe System
CLC Main Workbench 8	Qiagen Bioinformatics
FACS Diva V8.0.1	BD Bioscience
FlowJo V	Tree star Inc

Microsoft Office 2011	Microsoft
Prism8	GraphPad Software
BioRender	BioRender

2.2. Methods

2.2.1. Experimental treatment of mice

Intravenous injections were performed after heating mice under a red lamp and by injection into the tail vein of mice. Cells as well as reagents for intravenous injections were adjusted in PBS to be able to inject a total volume of 150 µl per intravenous injection. For intratracheal injections the mice were anesthetized using isoflurane and a total volume of 50 µl of reagents was injected into the lung using a cannula. After injection of the solution, the mice were ventilated to ensure an equal distribution of liquid in the lung. All reagents were adjusted with PBS to ensure that 50 µl could be injected intratracheal. For cell labeling in the lung 10 µM PKH26 were used. For MAIT cell activation 0.684 nmol or 100 nmol 5-A-RU/MeG mixture was used. For intratracheal immunizations 50 µg OVA and 5 µg CpG were used. For intravenous immunizations 200 µg OVA and 20 µg CpG as well as 50 nmol 5-A-RU/MeG mixture were used.

2.2.2. Preparing single cell suspensions

2.2.2.1. Lung

After euthanizing the mice, the lungs were taken out and placed in digestion medium. The tissue was cut in small pieces and incubated 20 min, shaking at 37 °C. After resuspending the remaining tissue, the cell suspension was incubated for another 20 min, shaking at 37°C. After filtering the single cell suspension through a 100 µm mesh, red blood cell lysis (RCB lysis) was performed using red blood cell lysis buffer for 3 min at room

temperature. The cells were washed by adding FACS buffer and followed centrifugation (400 g, 4 min, 4 °C). Now the cells were used for flow cytometry or cell isolation using MACS kits or sorting.

2.2.2.2. Spleen

Spleens were meshed trough a 70 or 100 µm strainer using a syringe plunger (2 ml). After centrifugation (400 g, 4 min, 4°C), RCB lysis was performed for 3 min at room temperature using 2 ml RCB lysis buffer followed by washing with FACS buffer.

2.2.2.3. Lymph node and thymus

The lymph nodes or thymus were placed in medium and grained using autoclaved frosted microscope slides followed by centrifugation (400 g, 4 min, 4°C). For human thymus samples three pieces of the whole organ were used for single cell suspensions.

2.2.2.4. Liver

The liver was perfused using 2 ml PBS at room temperature. Then it was meshed trough a 100 µm strainer and cells were washed. RCB lysis was performed for 3 min at RT. Afterwards, the cells were separated using a 40 %/80 % discontinuous percoll gradient. Therefore liver cells were resuspended in 40 % (v/v) percoll solution that was underlaid by 80 % (v/v) percoll solution. The gradient was centrifuged with 1400 g for 20 min with acceleration 7 and braking 1 at RT. After centrifugation the interphase was collected and cells were centrifuged and resuspended in FACS buffer.

2.2.3. Isolation of primary cells

2.2.3.1. T cell isolation

For T cells isolation, $CD8^+$ or $CD4^+$ T cell Isolation kits (Miltenyi) were used to perform a negative enrichment of $CD8^+$ and $CD4^*$ T cells. The spleen was prepared as explained above and cells were

counted to determine the cell number. All cells were used for T cell isolation. The isolation was performed according to manufactures instructions. This means cells were washed in MACS buffer and mixed with CD8α$^+$ T Cell biotin-antibody cocktail and incubated for 5 min at 4°C. Then MACS buffer was added and α-CD44 biotin was added and the cells were incubated for an additional 10 min at 4°C. Afterwards cells were washed by adding MACS buffer and followed centrifugation. Afterwards biotin microbeads were added to washed cells. After 15 min incubation at 4°C cells were applied to magnetic column and the flow-through was collected, which contained the enriched CD8$^+$ or CD4$^+$ T cells. The isolate cells were washed by adding PBS and followed centrifugation. After washing cells were counted and the isolated T cells were then used for cell transfer or *in vitro* cell culture.

2.2.3.2. DC isolation

For DC isolation the CD11c positive selection kit (Miltenyi) was used. Cells were isolated from tissue as described before. Then the isolation was performed according to manufactures instructions. In short, the cells were counted and washed by adding MACS buffer followed by centrifugation (300 g, 10 min, 4 °C). Then CD11c microbeads were added to the cells and incubated 15 min at 4°C. After washing the cells, they were applied to a LS column in a MACS seperator. After washing, the column was removed from the magnet and the CD11c$^+$ cells were flushed out from the column by adding MACS buffer and pushing the provided plunger into the column. The isolated cells were then used for RNA isolation and *in vitro* cell culture.

2.2.4. *In vitro* cell culture

For cell culture, T cells were isolated via T cell isolation kit (Miltenyi) as described above or via sorting. After isolation the cells were

seeded onto a 96-well flat-bottom plate, which was coated with α-CD3 (1 µg/ml). For murine MAIT cell stimulation, the MAIT cells were seeded in complete medium supplemented with α-CD28 (0.5 µg/ml) and IL-2 (100 U/ml). For human MAIT cells the complete medium was supplemented with α-CD28 (0.5 µg/ml), IL-2 (100 U/ml) and when stated with IL-7 (50 ng/ml) and PHA (3 µg/ml). For *in vitro* activation assays of MAIT cells using 5-A-RU/MeG a maximal concentration of 150 nM in complete medium was used.

2.2.5. *In vivo* cytotoxicity assay

Splenocytes were pulsed with the OVA peptide SIINFEKL (2 µg/ml) or not pulsed for 20 min at 37 °C. Cells were centrifuged and labeled with 0.4 µM CFSE (SIINFEKL pulsed cells - CFSElo) or 4 µM CFSE (non pulsed cells - CFSEhi) for 12 min at 4 °C. For stopping CFSE staining reaction, 10 % FCS was added followed by a washing step of the cells. Both target cell types (2x10^6 cells each), were injected intravenously. After 4 h, the target cells were analyzed in different organs using flow cytometry. The specific kill was determined using following formula.

$$\%\ \text{specific kill} = \left(1 - \frac{CFSElo\ primed}{CFSEhi\ primed} \Big/ \frac{CFSElo\ control}{CFSEhi\ control}\right) x100$$

2.2.6. Tetramer assembly

MR1-tetramers were generated from biotinylated MR1 monomers loaded either with 5-OP-RU or 6-FP (Reantragoon *et al.*, 2013; Corbett *et al.*, 2014; Eckle *et al.*, 2014; Eckle *et al.*, 2015). The monomers were tetramerised using streptavidin-PE (BD Bioscience), streptavidin-AF647 (Biolegend) or streptavidin-BV421 (Biolegend) at a 5:1 (monomer:streptavidin) molar ratio. Fluorochrome conjugated streptavidin was added sequentially by adding 1/10 of volume streptavidin at a series of 10 min incubations at 4°C. Besides in-house generated MR1 tetramers, mouse MR1-5-

OP-RU tetramers labeled with PE or APC were provided by the NIH tetramer core facility.

2.2.7. Flow cytometry

2.2.7.1. Staining of surface molecules

For analyzing surface molecules via flow cytometry single cell suspensions were resuspended in a mixture of antibodies and FcBlock that were diluted in FACS buffer. The cells were incubated for 30 min on ice and were washed twice with FACS buffer afterwards (400 g, 4 min, 4 °C). The cells were analyzed using the flow cytometer by resuspending the pellet in FACS buffer. All human samples were fixed using Fixation/ Perm solution from the FoxP3 staining kit (eBioscience) prior analysis at the flow cytometer.

2.2.7.2. Intracellular staining

Single cell suspensions were prepared and stained for surface molecules as described above. For transcription factor staining the FoxP3 staining Kit (eBioscience) was used. After staining of the surface molecules and washing the cells, they were resuspended in 1 ml Fixation/ Perm solution that was prepared according to manufactures instructions. For fixation the cells were incubated for 30 min on ice. Afterwards they were washed twice using Perm buffer. After the last washing step the cells were resuspended in intracellular antibody mixture and incubated for 30 minutes on ice. Finally the cells were washed twice using Perm buffer and resuspended in FACS buffer for measuring the cells at the flow cytometer.

For intracellular cytokine staining, BD Cytofix/Cytoperm kit (BD Biosciences) was used as per manufacturers instructions. Therefore the cells were resuspended in 500 µl Cytofix after surface staining and incubated for 30 minutes on ice. Afterwards the cells

were washed twice with Perm buffer and stained for intracellular cytokines at 4 °C for 30 min. Finally, the cells were washed twice using Perm buffer and resuspended in FACS buffer for measuring the cells at the flow cytometer.

2.2.8. Cytometric bead array (CBA)

Cell culture supernatants were collected and analyzed using CBA Flex Sets according to the manufactures instructions. Therefore 5 µl supernatant were mixed with 0.1 µl capture beads of each analyzed cytokine in a total volume of 5 µl. After incubation for 1 hour at room temperature in the dark, PE detection reagent was added. Therefore 0.1 µl PE detection reagent of each analyzed cytokine were added and topped up with diluent to a volume of 5 µl, which were added to the supernatant- bead mixture. After incubation for 1 hour at room temperature in the dark, FACS buffer was added and the plate was centrifuged. After an additional wash with FACS buffer the beads were resuspended in FACS buffer and analyzed at the flow cytometer. For each array a standard curve was prepared.

2.2.9. Multiplex PCR and TCR sequencing

After single cell sorting, cDNA was synthesized by adding 2.02 µl of master mix containing

	Amount [µl]
5x VILO reaction mix	0.5
10x Superscript RT	0.2
1% Triton X-100	0.22
Nuclease-free water	1.2

Temperature [°C]	Time [min]
25	10
42	120
85	5
16	Hold

After cDNA synthesis, two rounds of nested PCR were performed. For the first round, 23 µl of master mix was added to 2 µl of cDNA. For the 2nd round PCR, 2 µl of the 1st round product was mixed with 23 µl master mix containing either primer for α chain genes or β chain genes.

Round 1	Amount [µl]
GoTaq 2x master mix	12.5
TRAV multiplex fwd external primers (5 µM)	0.5
TRBV multiplex fwd external primers (5 µM)	0.5
TRAC reverse external primer (5 µM)	0.5
TRBC reverse external primer (5 µM)	0.5
Nuclease-free water	8.5

Round 2	Amount [µl]
GoTaq 2x master mix	12.5
TRAV or TRBV multiplex fwd internal primers (5 µM)	0.5
TRAC or TRBC reverse internal primer (5 µM)	1.0
Nuclease-free water	9

Temperature [°C]	Time [min]	
94	5	
94	30 s	35 cycles
52	30 s	
72	1	
95	1	
52	1	
72	7	
16	Hold	

After the 2nd PCR, presence of product was confirmed using 1.5% agarose gel. The PCR products were cleaned of remaining primers and nucleotides by using ExoSAP-IT. Therefore 5 µl PCR product was used and 1 µl ExoSAP-IT was added and incubated for 30 minutes at 37°C followed by 15 minutes at 80°C.

Cleaned PCR products were either sent to the company argf for sequencing evaluations at this stage or sequencing reaction was performed in house. For sequencing reaction by argf, 4 µl nuclease-free water and 2 µl primer (TRAC or TRBC internal) were added and plates were send of to argf. For sequencing reaction in house, 14 µl of mastermix was added to the 6 µl cleaned PCR product and the following PCR program was used.

	Amount [µl]
Nuclease-free water	6
DMSO	1
5x Dilution buffer	5
Internal reverse primer (TRAC or TRBC) (5µM)	1
BigDye	1

Temperature [°C]	Time [min]	
95	5	
96	10 s	} 35 cycles
50	5 s	
60	4	
16	Hold	

Afterwards 126 µl 85% ethanol and 4 µl 3 M NaOAc (pH5.3) were added and the plate was incubated 30 minutes at -20°C. Then the plate was centrifuged 45 minutes at 2200 g, 4°C. The liquid was removed and the pellet was resuspended in 150 µl 70% ethanol. The plate was centrifuged 45 minutes at 2200 g, 4°C and the liquid was removed afterwards. Then the dried pellet was send to argf for sequencing.

2.2.10. RNA isolation

After pelleting the cells by centrifugation, RNA was isolated using the isolation kit DNA, RNA and protein purification kit (Machery-Nagel) according to manufactures instructions. The RNA was eluted in 20 µl RNase-free water.

2.2.11. qRT-PCR

After RNA isolation cDNA was synthesized by adding 6.8 µl of mastermix to 1 µg RNA. The final volume of reaction mix was 20 µl and the reaction was done using the following PCR program.

Reverse Transcription:

	Amount [µl]
RT buffer	2
25x dNTPs	0.8
Random Primer	2
Reverse Transcriptase	1
RNase Inhibitor	1

Temperature [°C]	**Time [min]**
25	10
37	120
85	5
4	Hold

RT-PCR:

For qRT-PCR, mastermix was prepared as described below. Final reaction volume was 10 µl and the followed PCR program was used.

	Amount [µl]
DEPC	3
Primer fwd (50 µmol)	0.5
Primer rev (50 µmol)	0.5
SYBR Green	5
cDNA	1

Program:

Temperature [°C]	Time [min]	
50	2	
95	10	
95	15 sec	40 cycles
57	1	
72	1	
50	1 sec	
95	1 sec	
40	Hold	

2.2.12. Generation of TCR plasmid and transfection

For generation of TCR plasmids, specific, customized TCR sequences were obtained from Thermo Fisher. They were cloned into a 2A-peptide-linker pMIG expression vector using the restriction enzymes EcoRI and BgIII. Digestion with restriction enzymes was performed for 2 h at 37 °C. For the last 30 min of digestion antarctic phosphatase was added. Cut DNA fragments were purified with a 1 % agarose gel and the Wizard SV Gel and PCR Clean-up system. The cut and purified DNA fragments were used for ligation using T4 ligase with 20 ng plasmid and 80 ng insert. The ligation was performed over night at 4 °C. After ligation, the vector was transformed into *E.coli.* DH5α. Therefore, DNA was added to bacteria and the mixture of DNA and bacteria was kept on ice for 30 min, followed by 90 seconds at 42 °C. Bacteria were shook at 37 °C for 1 h. After transformation, bacteria were plated on agar-plates containing ampicillin for selection of bacteria that contain the plasmid. From grown colonies, two colonies were picked and grown over night in 10 ml LB medium supplemented with 100 µg/ml ampicillin. On the next day a minipreperation was performed to isolate the plasmid from bacteria. Therefore, ZymoPURE Plasmid Miniprep System Kit was used according to manufactures instructions.

The isolated plasmid was then used to transiently transfect a HEK293 T cell line. One day prior to transfection, cell were seeded in 24 well plates with 8 × 10^4 cells/well in RPMI medium. On the next day, medium was replaced with fresh RPMI medium. OptiMEM medium was mixed with Fugene HD and incubated for 10 min at RT. The OptiMEM/Fugene mix was added to the DNA mix with a final amount of 1.1 µg of DNA and incubated for 15 min at RT. The mixture was added dropwise to the HEK293 T cells. Afterwards the cells were incubated for 2 days at 37 °C, 5 % CO_2. 2 days after transfection, the cells were analyzed for MR1-tetramer reactivity as well as CD8 expression using flow cytometry.

2.2.13. Statistical analysis

All graphs and statistics analysis was performed using the program Prism8 (GraphPad). Scatter and bar graphs show mean ± SD. Statistical tests utilized Kruskal-Wallis test unless otherwise stated.

3. Chapter 3: Modulation of dendritic cell migration and activation by MAIT cells

3.1. Introduction

MAIT cells are a population of unconventional T cells located within various tissues throughout the body of mice and humans. In the lung, the liver and lamina propria the highest frequency of MAIT cells can be found in mice (Rahimpour *et al.*, 2015). In contrast, human MAIT cells are most abundant in liver (Dusseaux *et al.*, 2011). After the discovery that MAIT cells are activated by some, but not all bacteria (Le Bourhis *et al.*, 2010), it was shown that these responses were dependent on the presence of microbial metabolites produced during the biosynthesis of riboflavin (Kjer-Nielsen *et al.*, 2012). The most potent MAIT cell agonist currently identified is 5-OP-RU (5-(2-oxopropylideneamino)-6-D-ribitylaminouracil), which is derived from a non-enzymatic reaction of methylglyoxal with 5-A-RU (5-amino-6-D-ribitylaminouracil), an intermediate formed during riboflavin biosynthesis (Corbett *et al.*, 2014). It was shown that MAIT cells can be stimulated either *in vitro* using 5-OP-RU (Rahimpour *et al.*, 2015) or by injection of 5-OP-RU (Chen *et al.*, 2016), which made it possible to analyze their function without the context of infection. However, injection of 5-OP-RU alone did not lead to proliferation of MAIT cells, but adding a TLR ligand caused proliferation of MAIT cells *in vivo*, while TLR alone did not activate MAIT cells and did not induce proliferation of MAIT cells (Chen *et al.*, 2016).

Furthermore, 6-FP was shown to bind to MR1, but failed to activate MAIT cells (Kjer-Nielsen *et al.*, 2012), even in the presence of a TLR ligand (Chen *et al.*, 2016). Due to the described antigenic-specificity of MAIT cells, MR1-5-OP-RU tetramers are considered the most definitive means of identifying MAIT cells, while MR1-6-FP tetramers are commonly used as negative controls (Eckle *et al.*, 2014; Rahimpour *et al.*, 2015) due to antigen specificity.

Upon TCR stimulation, MAIT cells rapidly secrete an array of cytokines. In mice, this results in the secretion of pro-inflammatory cytokines such as IL-17A and IFNγ (Rahimpour *et al.*, 2015), while human MAIT cells mainly produce IFNγ and TNFα with only minor populations of MAIT cells producing IL-17A (Dusseaux *et al.*, 2011). Cytokines can act on other immune cells and can have an immunomodulatory effect within the immune system by suppressing or initiating immune responses as well as differentiating T_H cells (Holdsworth and Can, 2015).

At present, only a few studies show a direct link between MAIT cells and the modulation of immune cell function. For example, during *Francisella tularensis* infection in mice it was shown that MAIT cell dependent GM-CSF production promoted the differentiation of monocytes into DCs. Interestingly, here it was not shown directly that MAIT cells are the source of GM-CSF (Meierovics and Cowley, 2016). Furthermore, *in vitro* studies with human MAIT cells showed that they are also able to induce DC maturation. Co-cultures of MAIT cells with immature human DCs, induced upregulation of CD86, CD80, CD40 and PD-L1 on DCs in the presence of 5-A-RU/MeG in an MR1-dependent manner. Furthermore, IL-12 production by DCs was dependent on MR1 and on CD40L (Salio *et al.*, 2017), but presently no *in vivo* studies on the effect of MAIT cells on DCs exist.

DC maturation is induced by several factors. The most prominent factors include TLR ligands, like the TLR4 ligand lipopolysaccharide (LPS) (Rescigno *et al.*, 1999) and the TLR9 agonist CpG (unmethylated CpG DNA sequence) (Häcker *et al.*, 1998; Akbari *et al.*, 1999). Moreover, the local environment including cytokines plays an important role during maturation. Upon maturation, DCs upregulate co-stimulatory molecules and migrate to the draining lymph nodes (Banchereau *et al.*, 2000). Additionally, DCs upregulate chemokine receptors and start producing chemokines upon activation and during the maturation process (Sallusto *et al.*, 1999). Chemokines can direct migration of DCs on the one hand

but can also attract other immune cells like T cells on the other hand. Chemokine receptor 7 (CCR7) that is involved in homing of lymphocytes as well as mature DCs to lymphoid tissues (Förster *et al.*, 2008), is upregulated on DCs leading to migration into the LN (Sallusto, Schaerli, *et al.*, 1998; Förster *et al.*, 1999).

Upon activation of NKT cells, it was shown that DCs produce CCL17 as well as CCL22 in order to recruit $CD8^+$ T cells (Semmling *et al.*, 2010). NKT cells are furthermore able to induce DC maturation (Fujii *et al.*, 2003). Since NKT and MAIT cells have common features, like a rapid cytokine response upon stimulation or semi-invariant T cell receptors that recognize non-peptides, also MAIT cells might theoretically be able to affect DCs, as supported using human MAIT cells *in vitro* (Salio *et al.*, 2017).

Therefore, the aim of this study was to investigate the role of MAIT cell stimulation on DC activation in an *in vivo* setting. MAIT cells were activated in the lung by intratracheal injection of 5-A-RU/MeG and DCs were analyzed in the lung and in the lung-draining mediastinal lymph node (medLN) for their migratory behavior, as well as their maturation status and chemokine expression patterns.

3.2. Results

3.2.1. Activation of MAIT cells *in vivo* and *in vitro*

The microbial metabolite 5-OP-RU could activate MAIT cells *in vitro* as well as *in vivo* (Rahimpour *et al.*, 2015; Chen *et al.*, 2016). Furthermore, 5-A-RU mixed with methylglyoxal (MeG) could activate MAIT cells, since 5-A-RU and MeG react to 5-OP-RU (Soudais *et al.*, 2015). To validate if 5-A-RU used in this thesis was able to activate MAIT cells, 5-A-RU was mixed with MeG in a 1:1 ratio prior to intratracheal application. 4 h after intratracheal application of 5-A-RU/MeG, the activation markers CD25 and CD69 were measured on MAIT cells. Using MR1-5-OP-RU tetramers, MAIT cells were identified in the lung of mice (Fig. 3.1 A).

Additionally, it was shown that injection of 0.684 nmol and 100 nmol 5-A-RU with MeG caused around 40% of MAIT cells to express the activation markers CD69 and CD25 compared to PBS treated controls that did not upregulated CD25 and CD69. Only 0.2 % of MAIT cells expressed both activation markers, CD25 and CD69, after injection of PBS. Besides this, conventional T cells were not activated by injection of the MAIT cell antigen 5-A-RU/MeG. Additionally, a reduction of MAIT cell numbers was observed after injection of 5-A-RU/MeG, which could be due to TCR internalization upon stimulation of MAIT cells and prevention of MR1-5-OP-RU tetramer binding to TCR.

Besides *in vivo*, the effect of 5-A-RU/MeG was analyzed *in vitro* on whole splenocytes by assessing the proliferation of MAIT cells labeled with cell trace violet (CTV) whose dilution indicates cell divisions. As shown in figure 3.1 B, no proliferation was observed, when cells were not stimulated. Adding the antigen 5-A-RU/MeG, proliferation was observed in a dose-dependent manner. Purified 5-OP-RU, the reaction product of 5-A-RU and MeG, was used as positive control (Rahimpour *et al.*, 2015) and induced the highest frequency and proliferation of MAIT cells. This indicated that MAIT cells could be stimulated *in vitro* using 5-A-RU/MeG, but 5-OP-RU led to higher MAIT cell proliferation than 5-A-RU/MeG indicating a higher activation of MAIT cells using 5-OP-RU. This could be due to an incomplete reaction of 5-A-RU with MeG or 5-A-RU that was already degraded due to high instability.
Because pure 5-OP-RU was not available in sufficient amounts, 5-A-RU mixed with MeG was used for further studies since it is also efficient to activate MAIT cells *in vivo*.

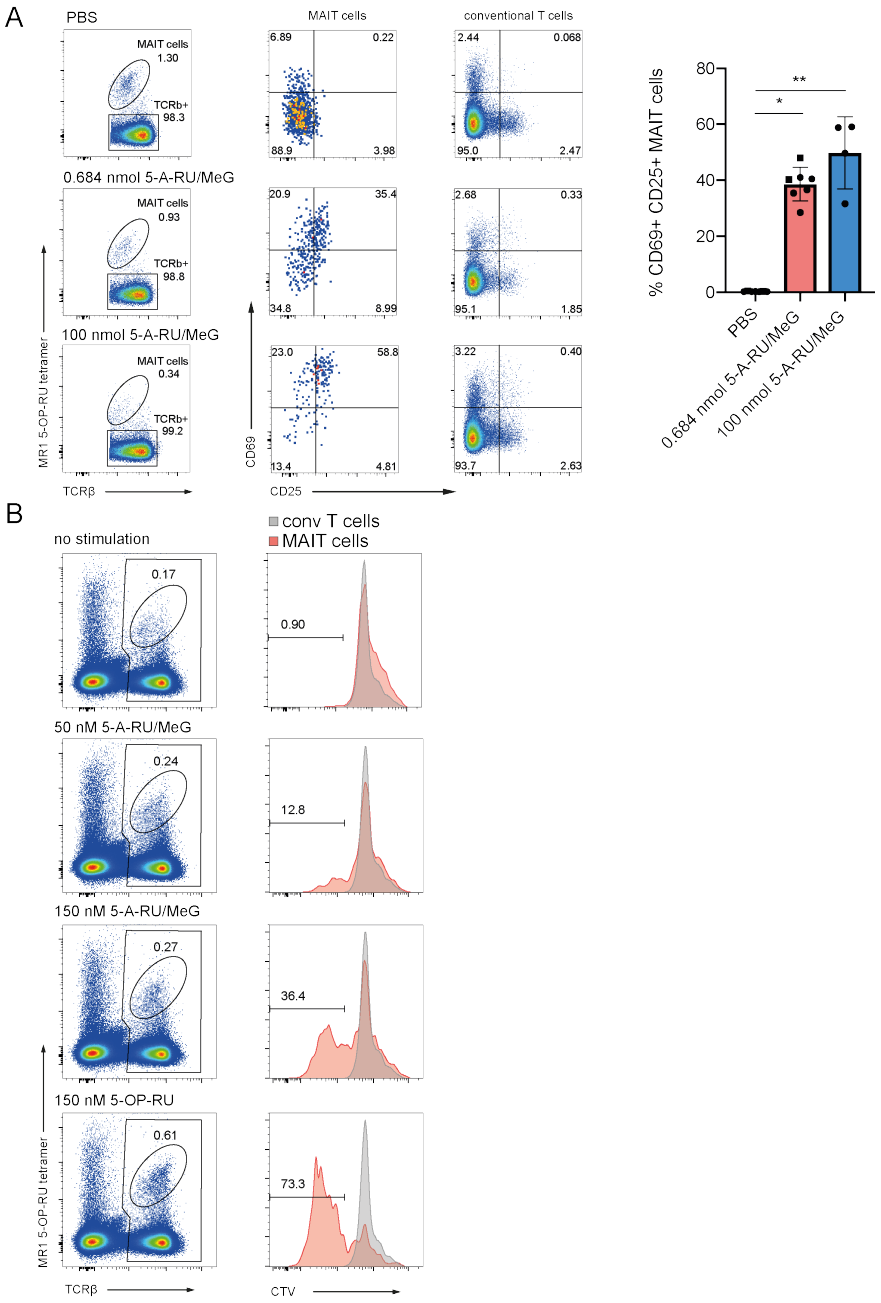


Figure 3. 1 – Activation of MAIT cells *in vivo* and *in vitro.* (A) Mice were treated with PBS, 0.684 nmol or 100 nmol 5-A-RU/MeG intratracheally. After 4 h, the activation of MAIT cells in the lung was assessed by flow cytometry. The dot plots are representative and show the gating of MAIT cells as well as the activation markers CD25 and CD69. The bar graph shows $CD25^{+}$ $CD69^{+}$ MAIT cells from all MAIT cells of three independent experiments. Squared dots are obtained from an experiment with 5-A-RU/MeG kindly provided by Olivier Lantz. Round dots show data created by usage of 5-A-RU generated at the University Bonn in the laboratory of Prof. Dr. Dirk Menche. (n=3 for every concentration) *P≤0.05, **P≤0.01 (B) *In vitro* stimulation of MAIT cells with 5-A-RU/MeG. Splenocytes were stimulated with the indicated concentrations of 5-A-RU/MeG or 5-OP-RU and proliferation of MAIT cells was assed via CTV. (n=1).

3.2.2. MAIT cell activation in the lung induce DC migration

Dendritic cells are important cells within the immune system. They encounter antigens in the periphery and transport them to the lymph nodes to present them to T cells. Since MAIT cells are rapidly activated after antigen stimulation and *in* vitro studies of human MAIT cells showed effects on DCs, the role of MAIT cells and their immunomodulatory effect on DCs was investigated. Therefore, 5-A-RU/MeG was applied intratracheal in the lungs of mice and DCs were analyzed 24 h after application in the lung and in the lung-draining mediastinal lymph node (medLN). Besides 5-A-RU/MeG, PBS was used as negative control as well as CpG as positive control for DC maturation. As shown in figure 3.2 A, the numbers of $CD11b^+$ DCs (cDC2s) increased 24 h after stimulation with either 5-A-RU/MeG or CpG in the lung even though only the difference between PBS and CpG treated mice was significant. In contrast, numbers of $CD103^+$ DCs (cDC1s) did not change at the same time point. In the medLN, the numbers of $CD11b^+$ DCs as well as $CD103^+$ DCs significantly increased after intratracheal stimulation with 5-A-RU/MeG or CpG compared to PBS treated mice (Fig. 3.2 B). This indicated that MAIT cell activation led to accumulation of $CD11b^+$ DCs in the lung. In the medLN more $CD103^+$ DCs were found after 5-A-RU/MeG and CpG treatment, suggesting that migration from the lung may occur. But also recruitment from other sites is possible. Besides $CD103^+$ DCs, also $CD11b^+$ DCs could be observed in higher numbers in the medLN upon stimulation with 5-A-RU/MeG and CpG compared to PBS treated mice. This suggested that also $CD11b^+$ DCs immigrated from the lung, but also in this case, recruitment from other sites is possible.

To directly test whether DC migration from the lung to the medLN was induced by MAIT cell activation, the cell membrane dye PKH26 was applied into the lungs of mice prior to activation of MAIT cells, which allowed tracking cell migration from the lung to the medLN. PKH26 has been used for migration studies in the lung before and was concluded

not to induce inflammation as observed for the dye CFSE (Nakano *et al.*, 2013). PKH26 was applied intratracheal prior to MAIT cell stimulation. 24 h after stimulation of MAIT cells, DCs were analyzed in the medLN. The experimental setup is shown in figure 3.3 A. As shown in figure 3.3 B, by applying a gate for $CD11c^+$ and $MHCII^+$ cells after exclusion of doublets, dead and autofluorescent cells, DCs were identified. Using CD103 as well as CD11b, the DCs were subdivided into the two conventional DC subsets, CD103+ DCs (cDC1) and CD11b+ DCs (cDC2). Then the different subsets were analyzed for PKH26 staining. Since the dye was injected into the lung, only cells from the lung should be positive for PKH26. So only cells that have migrated from the lung stained with PKH26 in the medLN. $CD45^-$ cells were negative for PKH26, indicating that no diffusion of PKH26 into the medLN occurred. As shown in figure 3.3 C, the numbers of $CD11b^+$ DCs increased upon stimulation with 5-A-RU/MeG or CpG. While the dose of 0.684 nmol 5-A-RU/MeG only showed a tendency of higher DC numbers, 100 nmol 5-A-RU/MeG treatment showed significant higher numbers of $CD11b^+$ DCs. Also $CD103^+$ DC numbers increased upon treatment with 5-A-RU/MeG and CpG with 100 nmol 5-A-RU/MeG and CpG showing significant differences compared to PBS control mice (Fig.3.3 D). Analyzing DC numbers of PKH26 positive cells, $CD11b^+$ $PKH26^+$ DCs showed significant increased numbers in 100 nmol 5-A-Ru/MeG treated mice. (Fig. 3.3 E). The numbers of $CD103^+$ $PKH26^+$ DCs also significantly increased upon stimulation with 100 nmol 5-A-RU/MeG similar to CpG treatment, while 0.684 nmol 5-A-RU/MeG stimulation only showed a tendency of higher numbers that is not significant (Fig. 3.3 F). This indicated that 5-A-RU/MeG induced migration of $CD11b^+$ DCs as well as $CD103^+$ DCs from the lung to the medLN.

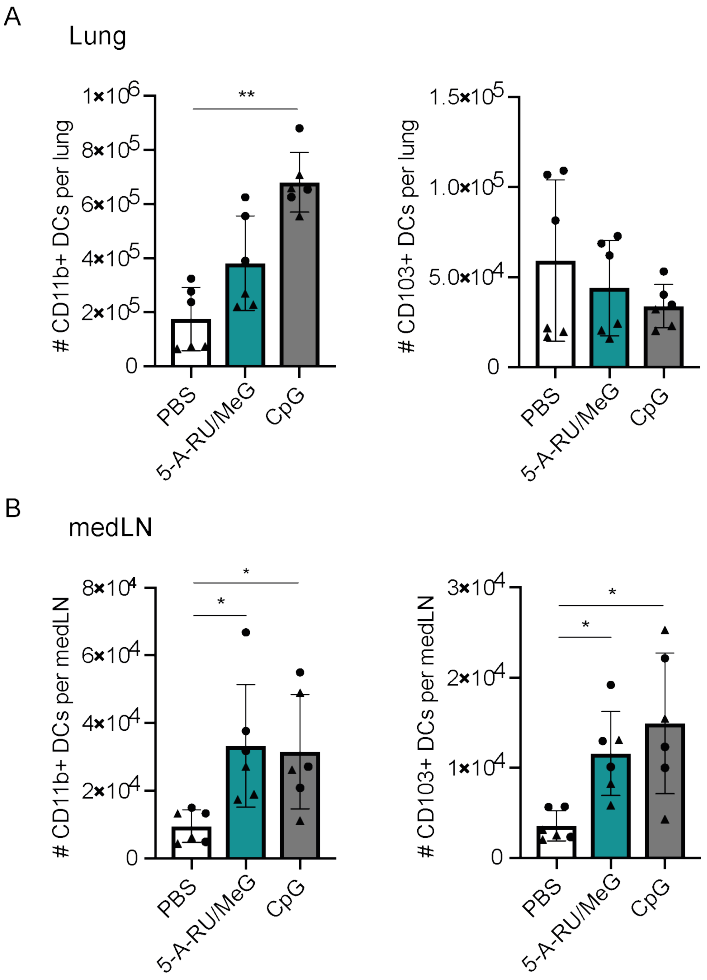


Figure 3. 2 – DC numbers in lung and medLN 24 h after stimulation with PBS, 5-A-RU/MeG or CpG in CCL17eGFP mice. (A) Cell numbers of $CD11b^+$ and $CD103^+$ DCs per lung 24 h after intratracheal injection of either PBS, 5-A-RU/MeG or CpG. (B) Cell numbers of $CD11b^+$ and $CD103^+$ DCs per medLN 24 h after intratracheal injection of either PBS, 5-A-RU/MeG or CpG. The data is combined from two independent experiments. Triangles and dots represent the different experiments *P≤0.05, **P≤0.01

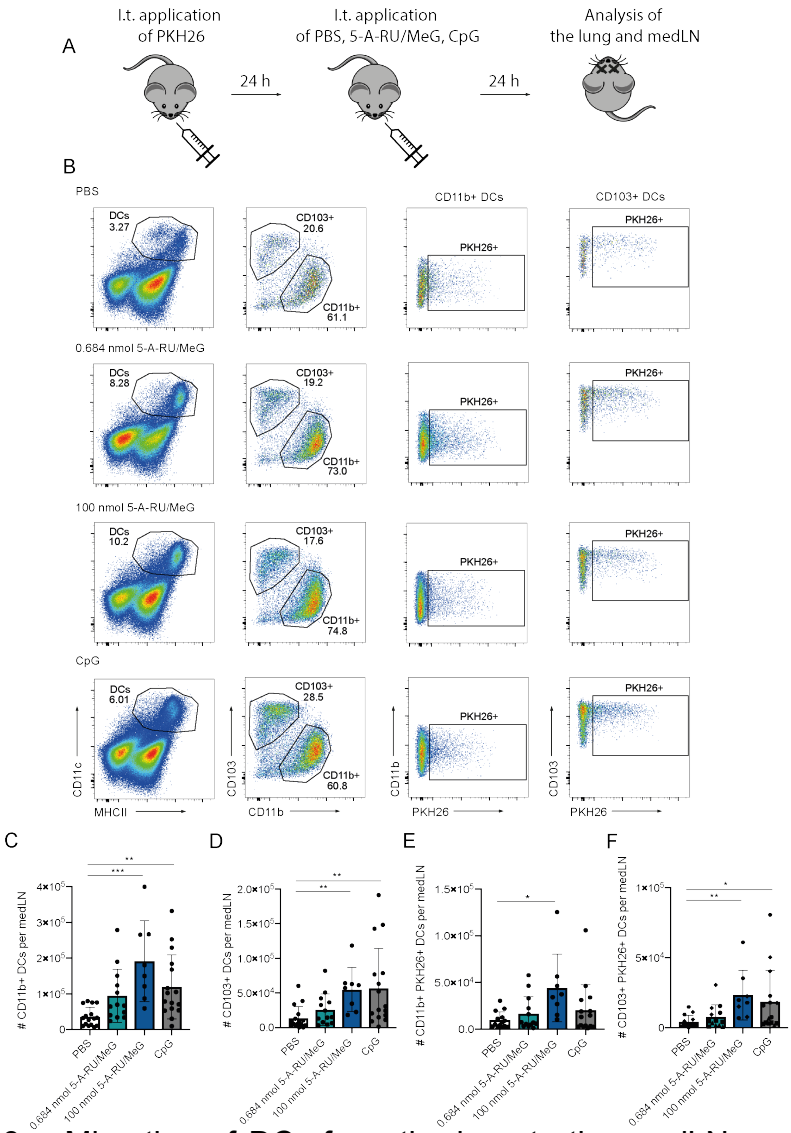


Figure 3. 3 – Migration of DCs from the lung to the medLN upon stimulation with 5-A-RU/MeG. (A) Experimental setup. PKH26 was administered intratracheally 24 h prior to intratracheal application of either PBS, 5-A-RU/MeG or CpG. 24 after stimulation the lungs and medLNs were analyzed for DC numbers. (B) Gating strategy. After gating on live, single CD45^{+} autofluorescence^{-} cells, DCs were gated using CD11c and MHCII. Further subdivision into the different DC subsets by the marker CD11b as well as CD103. The cells were then analyzed for PKH26 staining. (C) Absolute numbers of CD11b^{+} DCs per medLN after PKH26 injection and stimulation with either PBS, 5-A-RU/MeG or CpG. (D) Absolute numbers of CD103^{+} DCs per medLN after PKH26 injection and stimulation with either PBS, 5-A-RU/MeG or CpG. (E) Absolute numbers of CD11b^{+} PKH26^{+} DCs per medLN after PKH26 injection and stimulation with either PBS, 5-A-RU/MeG or CpG. (F) Absolute numbers of CD103^{+} PKH26^{+} DCs per medLN after PKH26 injection and stimulation with either PBS, 5-A-RU/MeG or CpG. The data is combined from four independent experiments for low dose 5-A-RU/MeG and two independent experiments for high dose 5-A-RU/MeG. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

Upon stimulation, DCs usually upregulate co-stimulatory molecules, that are important for inducing immunogenic responses. Therefore, analysis whether co-stimulatory molecules are upregulated on DCs upon stimulation of MAIT cells was performed. In the lung, CD86 was significantly upregulated on $CD11b^+$ DCs after application of 100 nmol 5-A-RU/MeG while no change was observed after administration of 0.684 nmol 5-A-RU/MeG. Significant upregulation of CD80 was observed on $CD11b^+$ DCs after stimulation with CpG as well as both doses of 5-A-RU/MeG with 100 nmol 5-A-RU/MeG leading to a higher expression of CD80. CD40 upregulation was seen on $CD11b^+$ DCs upon all stimulations. Stimulation with 0.684 nmol 5-A-RU/MeG led to a difference with a p-value of 0.0602 while the other stimulations led to a significant upregulation of CD40. In $CD103^+$ DCs, CD86 expression was increased upon all stimulations. Here, treatment with 100 nmol 5-A-RU/MeG or CpG led to significant upregulation of CD86. CD80 expression was unchanged after treatment with 0.684 nmol 5-A-RU/MeG and also the higher dose of 100 nmol 5-A-RU/MeG did not change CD80 expression significantly. In contrast, CpG treatment led to a significant upregulation of CD80. CD40 were significantly upregulated on $CD103^+$ DCs after stimulation with 100 nmol 5-A-RU/MeG as well as CpG. These results indicated that upregulation of co-stimulatory molecules on pulmonary DCs occurred upon activation of MAIT cells with 100 nmol of 5-A-RU/MeG while 0.684 nmol 5-A-RU/Meg only lead to an unclear activation of DCs.

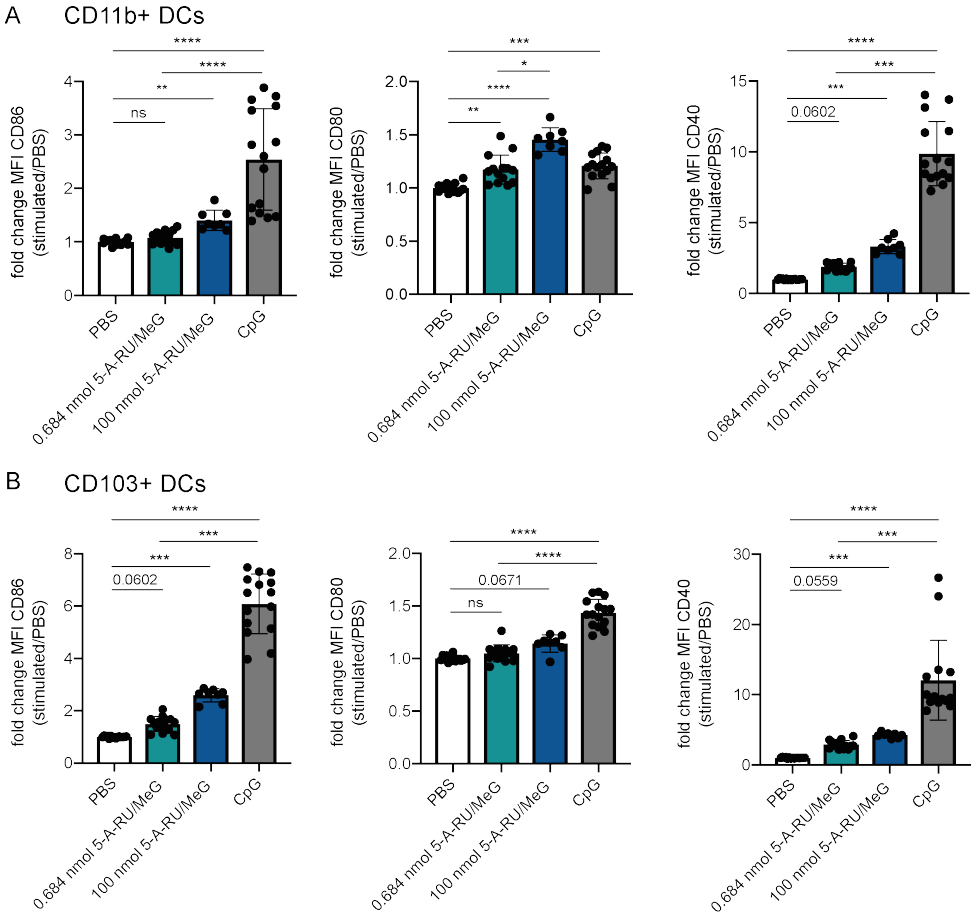


Figure 3. 4 - Expression of co-stimulatory molecules on the different DC subsets in the lung after treating C57BL/6J mice with PBS, 5-A-RU/MeG or CpG intratracheally. After intratracheal application of PKH26 and application of PBS, 5-A-RU/MeG and CpG afterwards, pulmonary DCs were analyzed for co-stimulatory molecule expression using flow cytometry. (A) Expression of CD80, CD86 and CD40 on $CD11b^+$ DCs 24 h after intratracheal application of PBS, 5-A-RU/MeG or CpG. The data is combined from four different experiments for low dose 5-A-RU/MeG and two independent experiments for high dose 5-A-RU/MeG (8 - 16 mice per group) by normalization of 5-A-RU/MeG or CpG treated groups to the mean of PBS treated mice from each experiment. (B) Expression of CD80, CD86 and CD40 on $CD103^+$ DCs 24 h after intratracheal application of PBS, 5-A-RU/MeG or CpG. The data is combined from four different experiments for low dose 5-A-RU/MeG and two independent experiments for high dose 5-A-RU/MeG (8 - 16 mice per group) by normalization of 5-A-RU/MeG or CpG treated groups to the mean of PBS treated mice from each experiment. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

In addition to pulmonary DCs, medLN DCs were analyzed for expression of co-stimulatory molecules. As shown in figure 3.5 A, $CD11b^+$ DCs only

showed significant upregulation of CD86 after stimulation with 0.684 nmol 5-A-RU/MeG, while CD80 and CD40 were not significantly upregulated. In contrast, application of 100 nmol 5-A-RU/MeG showed significant upregulation of CD86, CD80 and CD40 on $CD11b^+$ DCs. Also treatment with CpG compared to PBS treated control mice showed upregulation of CD86, CD80 and CD40. $CD103^+$ DCs upregulated CD86 after stimulation with 0.684 nmol 5-A-RU/MeG compared to PBS treated control mice, while CD80 and C40 were not upregulated with this dose of 5-A-RU/MeG. 100 nmol 5-A-RU/MeG application led to significant upregulation of CD86, CD80 and CD40. Also CpG treated mice showed upregulation of CD80 and CD86 and CD40 on $CD103^+$ DCs. These data indicated that MAIT cell stimulation could induce DC maturation, but in a dose dependent manner. 0.684 nmol 5-A-RU/MeG only showed upregulation of CD86 compared to PBS treated mice while 100 nmol 5-A-RU/MeG showed upregulation of all tested activation markers with a similar expression level to CpG treated mice. This could indicate that low dose MAIT cell activation leads to a partial maturation of DCs in the medLN, while high dose (100 nmol) of 5-A-RU/MeG could lead to a full DC maturation.

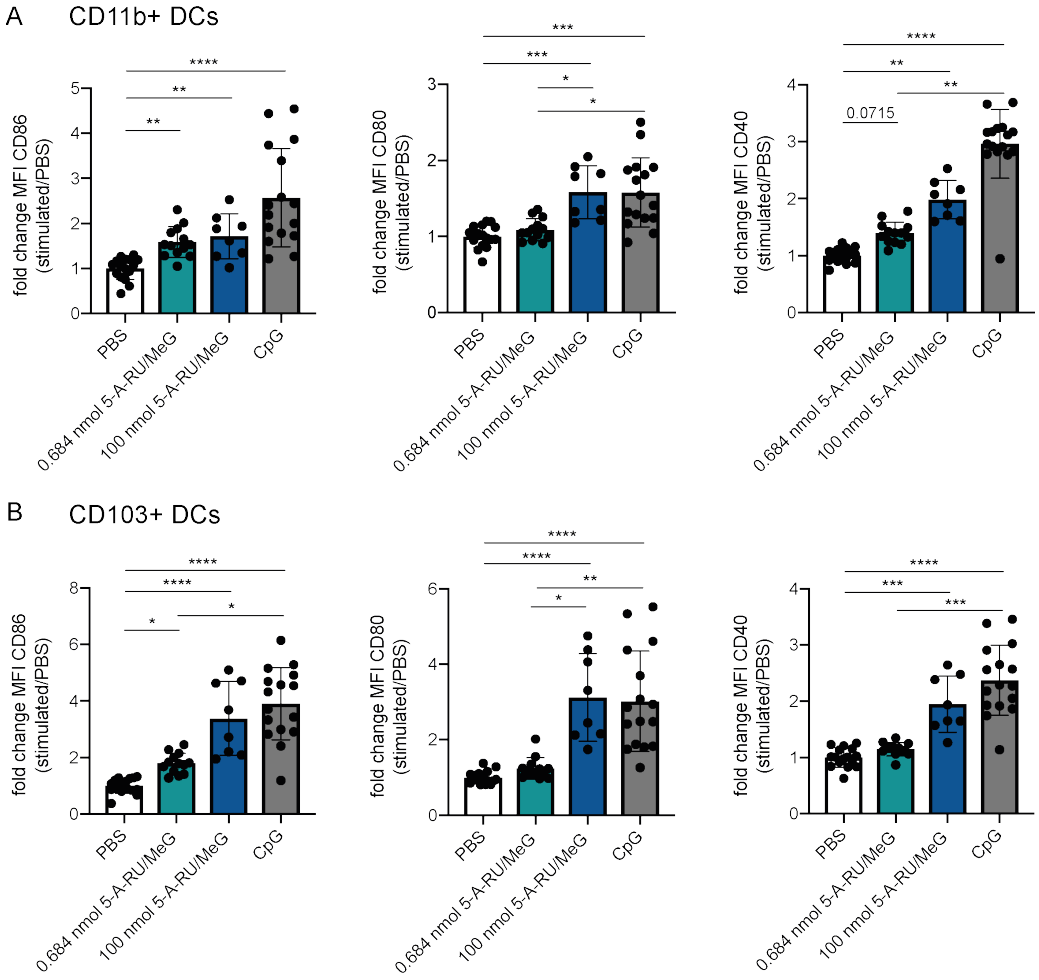


Figure 3. 5 – Expression of co-stimulatory molecules on the different DC subsets in the medLN after treating C57BL/6J mice with PBS, 5-A-RU/MeG or CpG intratracheally. After intratracheal application of PKH26 and application of PBS, 5-A-RU/MeG and CpG afterwards, the DCs from the medLN were analyzed for co-stimulatory molecule expression using flow cytometry. (A) Expression of CD86, CD80 and CD40 on $CD11b^+$ DCs 24 h after intratracheal application of PBS, 5-A-RU/MeG or CpG. The data is combined from four different experiments for low dose 5-A-RU/MeG and two independent experiments for high dose 5-A-RU/MeG (8 - 16 mice per group) by normalization of 5-A-RU/MeG or CpG treated groups to the mean of PBS treated mice from each experiment. (B) Expression of CD86, CD80 and CD40 on $CD103^+$ DCs 24 h after intratracheal application of PBS, 5-A-RU/MeG or CpG. The data is combined from four experiments for low dose 5-A-RU/MeG and two independent experiments for high dose 5-A-RU/MeG different experiments (8 – 16 mice per group) by normalization of 5-A-RU/MeG or CpG treated groups to the mean of PBS treated mice from each experiment. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

3.2.3. DC migration after activation of MAIT cells is MR1 dependent

To verify the previously reported findings that DC migration and DC maturation were due to MAIT cell activation, the experiment described above was performed with intratracheal PKH26 injection and followed MAIT cell stimulation in $MR1^{-/-}$ mice. $MR1^{-/-}$ mice lack MAIT cells because MR1 expression is necessary during MAIT cell development (Treiner *et al.*, 2003). Using $MR1^{-/-}$ mice, the experimental setup shown in figure 3.3 A was performed, where MAIT cells were stimulated after PKH26 injection. 24 h after MAIT cell stimulation DCs were analyzed in the lung and medLN. Analysis of pulmonary DCs showed that their percentage was unchanged in $MR1^{-/-}$ mice after stimulation with 5-A-RU/MeG compared to PBS stimulation. Dividing the DCs into the two different subsets showed that the frequency of $CD103^+$ DCs as well as $CD11b^+$ DCs was similar after injection of 5-A-RU/MeG compared to PBS treated mice (Fig. 3.6 A). This indicated that in $MR1^{-/-}$ the migration of $CD103^+$ DCs as well as accumulation of $CD11b^+$ DCs was impaired compared to C57BL/6J mice shown in appendix figure 1.

Calculation of the absolute numbers per lung showed that $CD11b^+$ DC numbers stayed unchanged in $MR1^{-/-}$ mice treated with 5-A-RU/MeG or PBS, while CpG treated mice showed significantly higher $CD11b^+$ DC numbers (Fig. 3.6 B). In contrast, the numbers of $CD103^+$ DCs were lower in $MR1^{-/-}$ mice treated with CpG compared to all other treatments, while numbers were unchanged when mice were treated with 5-A-RU/MeG (Fig. 3.6 C). This also suggested that the emigration of $CD103^+$ DCs was impaired in $MR1^{-/-}$ mice, whereas the recruitment of $CD11b^+$ DCs was defective. To investigate if activation of DCs is impaired in $MR1^{-/-}$ mice as well, the expression of CD86, CD80 as well as CD40 on DCs in the lung was analyzed. As shown in figure 3.7 A, expression of CD86, CD80 and CD40 was unchanged on $CD11b^+$ DCs in $MR1^{-/-}$ between PBS and 5-A-RU/MeG treated mice. In contrast, CpG treatment induced upregulation of CD86, CD80 as well as CD40 compared to PBS treated mice. Similar to $CD11b^+$ DCs, $CD103^+$ DCs

showed unchanged CD86, CD80 as well as CD40 expression comparing PBS treated to 5-A-RU/MeG treated mice. Only CpG induced upregulation of the co-stimulatory molecules on $CD103^{+}$ DCs in $MR1^{-/-}$ mice. This indicated that activation of DCs by MAIT cells in the lung is impaired in $MR1^{-/-}$ mice. Especially 100 nmol 5-A-RU/MeG did not change any activation marker on DCs in $MR1^{-/-}$, while this dose led to upregulation of CD86, CD80 and CD40 on both DC subsets in C57BL/6J mice (Fig. 3.4)

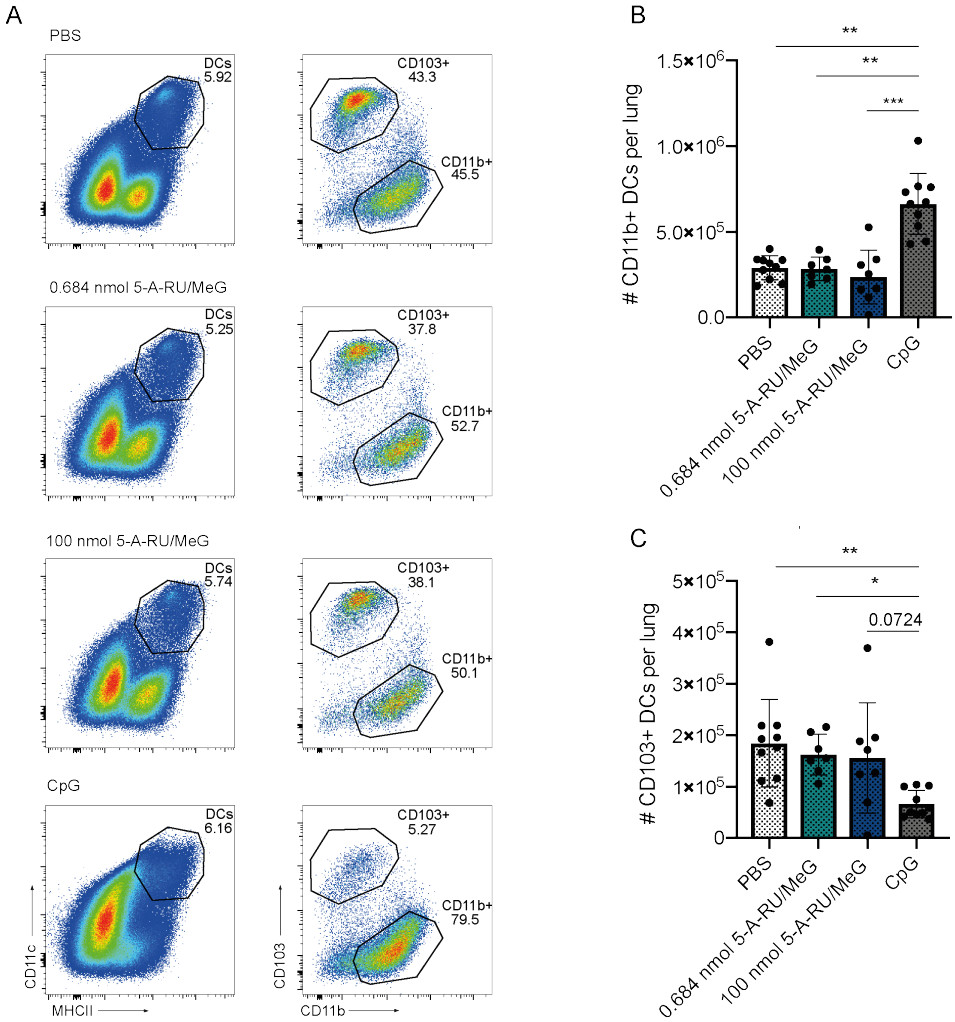


Figure 3. 6 – Effect of 5-A-RU/MeG on DCs in the lung of MR1$^{-/-}$ mice. (A) Gating of DCs and their subsets in MR1$^{-/-}$ mice after stimulation with PBS, 5-A-RU/MeG or CpG. (B) Absolute numbers of CD11b^{+} DCs in the lung of MR1$^{-/-}$ mice after different stimulations. (C) Absolute numbers of CD103^{+} DCs in the lung of MR1$^{-/-}$ mice after different stimulations. The data is combined from two different experiments for low dose of 5-A-RU/MeG and two different experiments for high dose of 5-A-RU/MeG with 3-4 mice per group per experiment. *P≤0.05, **P≤0.01, ***P≤0.001

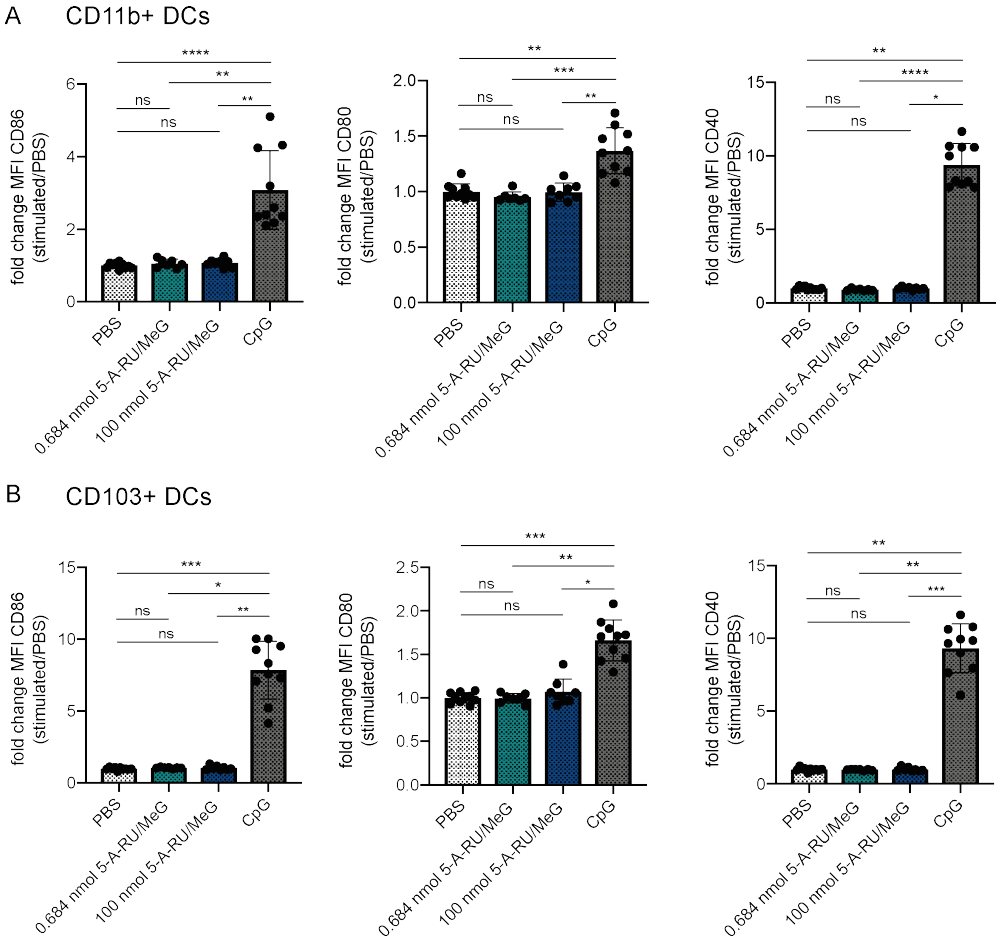


Figure 3. 7 – Expression of co-stimulatory molecules CD80, CD86 and CD40 on $CD11b^+$ and $CD103^+$ DCs in the lungs of $MR1^{-/-}$ mice after intratracheal injection of PKH26 and stimulation with PBS, 5-A-RU/MeG or CpG. PKH26 was applied intratracheally followed by intratracheal application of PBS, 5-A-RU/MeG or CpG 24 h later, followed by analysis of CD86, CD80 and CD40 expression 24 h after stimulation. (A) Expression of CD86, CD80 and CD40 on $CD11b^+$ DCs in $MR1^{-/-}$ mice. (B) Expression of CD86, CD80 and CD40 on $CD103^+$ DCs in $MR1^{-/-}$ mice. The data is combined from two different experiments for low dose of 5-A-RU/MeG and two different experiments for high dose of 5-A-RU/MeG with 3-4 mice per group per experiment. The data is combined by normalization of 5-A-RU/MeG or CpG treated groups to the mean of PBS treated mice from each experiment. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

Also in the medLN, a difference was observed between $MR1^{-/-}$ mice and C57BL/6 mice. As shown in figure 3.8 A, no difference in the frequency of DCs was observed in the medLN of $MR1^{-/-}$ mice after injection of 5-A-

RU/MeG compared to mice treated with PBS or CpG. Analyzing DC subsets, $MR1^{-/-}$ mice showed lower numbers of $CD11b^{+}$DCs, when treated with 0.684 nmol 5-A-RU/MeG compared to PBS control, while 100 nmol 5-A-RU/MeG or CpG treated mice showed numbers similar to PBS treatment (Fig. 3.8 B). Also lower numbers of $CD103^{+}$ DCs were observed in $MR1^{-/-}$ mice treated with 0.684 nmol 5-A-RU/MeG with differences being not significant (Fig. 3.8 C). Using PKH26 to track migratory cells, no changes in $CD11b^{+}$ $PKH26^{+}$ DC numbers in the medLN of $MR1^{-/-}$ mice were observed independent of treatment (Fig. 3.8 D). Moreover, $CD103^{+}$ $PKH26^{+}$ DC numbers were unchanged in the medLN of $MR1^{-/-}$ (Fig. 3.8 E). This indicated that DC migration is impaired in $MR1^{-/-}$ mice, if the DC numbers were compared to C57BL/6 mice shown in figure 3.3. This demonstrated that DC migration after injection of 5-A-RU/MeG was MR1- and MAIT-cell dependent.

Furthermore, activation markers were analyzed on DCs in the medLN. As shown in figure 3.9 A no difference in the expression of CD86, CD80 and CD40 was observed in $CD11b^{+}$ DCs of $MR1^{-/-}$ mice treated with PBS or 5-A-RU/MeG, while CpG treatment induced significant upregulation of CD86, CD80 and CD40 (Fig. 3.9 A). $CD103^{+}$ DCs also showed unchanged expression of CD80, CD86 as well as CD40 in 5-A-RU/MeG treated mice compared to PBS treated $MR1^{-/-}$ mice, while CpG cause significant upregulation of the tested activation markers. (Fig. 3.9 B). Comparison of $MR1^{-/-}$ mice to C57BL/6J mice showed that at least 100 nmol 5-A-RU/MeG induced upregulation of all activation markers on both DC subsets in C57BL/6 mice (Fig.3.5) while $MR1^{-/-}$ mice treated with 100 nmol 5-A-RU/MeG did not show a change in expression of activation markers compared to PBS treated mice. This indicated that besides DC migration, activation of DCs was dependent on MR1 and MAIT cells. Additional CpG treatment showed that activation of DCs in general was not impaired in $MR1^{-/-}$ mice.

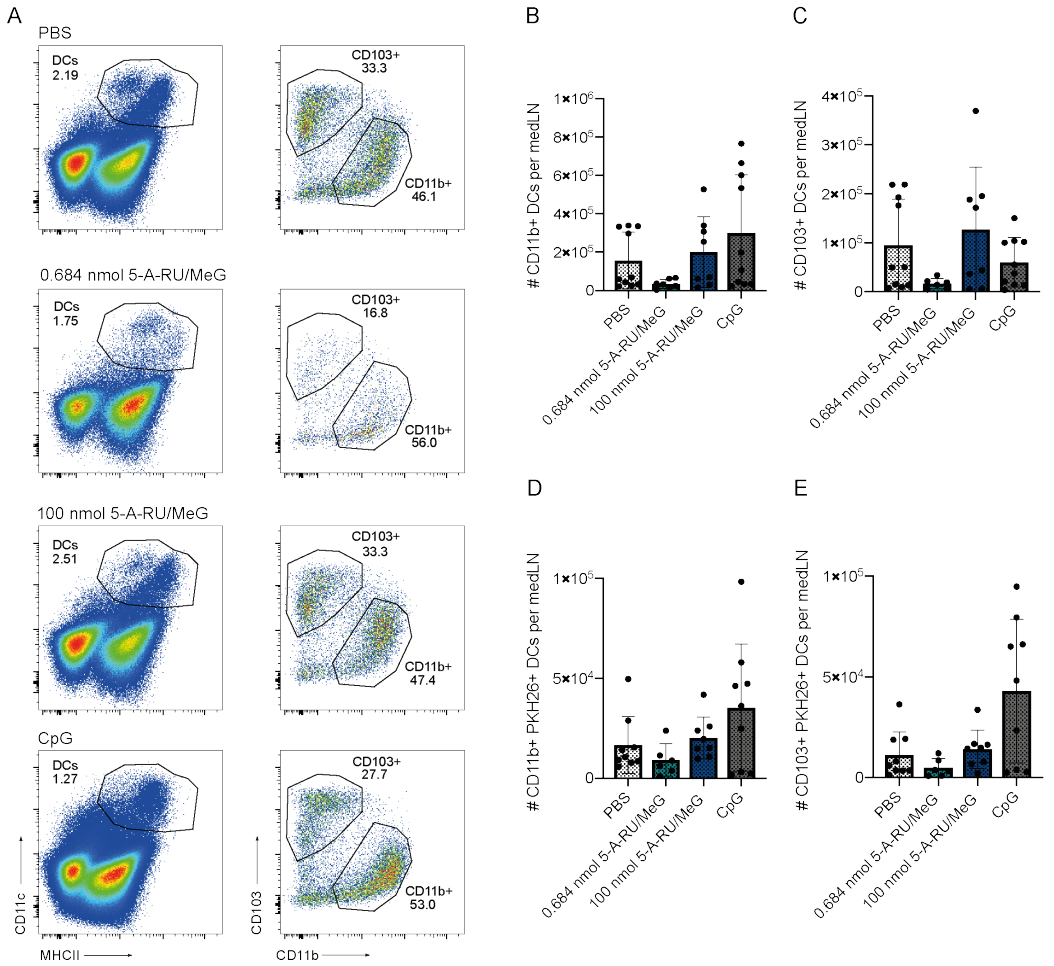


Figure 3. 8 - Effect of 5-A-RU/MeG on DCs in the medLN of MR1$^{-/-}$ mice. (A) Gating of DCs and their subsets in the medLN of MR1$^{-/-}$ mice after stimulation with PBS, 5-A-RU/MeG or CpG. (B) Absolute numbers of CD11b^{+} DCs in the medLN of MR1$^{-/-}$ mice after stimulation (C) Absolute numbers of CD103^{+} DCs in the medLN of MR1$^{-/-}$ mice after stimulation. (D) Absolute numbers of CD11b^{+} PKH26^{+} DCs in the medLN of MR1$^{-/-}$ mice after PKH26 injection and stimulation with PBS, 5-A-RU/MeG or CpG. (E) Absolute numbers of CD103^{+} PKH26^{+} DCs in the medLN of MR1$^{-/-}$ mice after PKH26 injection and stimulation with PBS, 5-A-RU/MeG or CpG. injection. The data is combined from two different experiments for low dose of 5-A-RU/MeG and two different experiments for high dose of 5-A-RU/MeG with 3-4 mice per group per experiment.

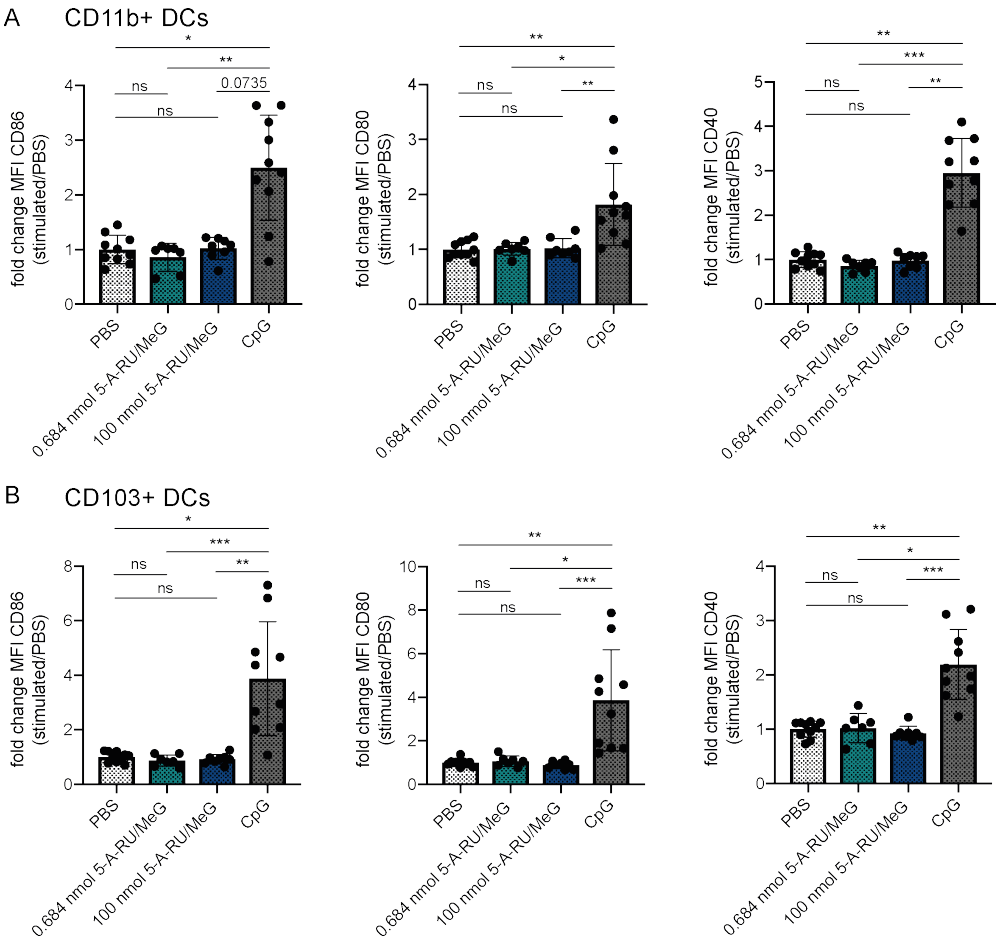


Figure 3. 9 - Expression of CD86, CD80 and CD40 on $CD11b^+$ and $CD103^+$ DCs in the medLNs of $MR1^{-/-}$ mice after intratracheal injection of PKH26 and PBS, 5-A-RU/MeG or CpG. PKH26 was applied intratracheally followed by intratracheal application of PBS, 5-A-RU/MeG or CpG 24 h later, followed by analysis of CD86, CD80 and CD40 expression 24 h after stimulation. (A) Expression of CD86, CD80 and CD40 on $CD11b^+$ DCs in $MR1^{-/-}$ mice. (B) Expression of CD86, CD80 and CD40 on $CD103^+$ DCs in $MR1^{-/-}$ mice. The data is combined from two different experiments for low dose of 5-A-RU/MeG and two different experiments for high dose of 5-A-RU/MeG with 3-4 mice per group per experiment. The data was combined by normalization of 5-A-RU/MeG or CpG treated groups to the mean of PBS treated mice from each experiment. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

3.2.4. DCs produce CCL17 and CCL22 after MAIT cell activation

DCs are able to recruit other immune cells by chemokine production upon stimulation and maturation. For example, it is known that DCs can produce CCL17 to recruit $CD8^+$ T cells in the context of cross-priming (Semmling *et al.*, 2010). After the finding that DCs migrate to the medLN and upregulate of co-stimulatory molecules, the production of chemokines by DCs was investigated. First, the production of CCL17 after 5-A-RU/MeG injection and MAIT cell activation was analyzed. To this end, the reporter mice that expressed an eGFP protein under the CCL17 promotor was used (Alferink *et al.*, 2003). As shown in figure 3.10 A, the percentage of $CCL17^+$ $CD11b^+$ DCs did not significantly change in the lung upon stimulation with 5-A-RU/MeG, but significantly decreased after stimulation with CpG. Analyzing $CD103^+$ DCs for expression of CCL17 in the lung, a significant higher frequency of $CCL17^+$ $CD103^+$ DCs were observed upon intratracheal stimulation with 0.684 nmol 5-A-RU/MeG. Treatment with CpG led to a higher frequency of $CCL17^+$ $CD103^+$ DCs with a statistic p-value of 0.0513, while treatment with 100 nmol 5-A-RU/MeG did not led to a significant higher frequency of $CCL17^+$ $CD103^+$ DCs (Fig. 3.10 B). In the medLN a significant higher frequency of $CCL17^+$ $CD11b^+$ DCs was observed when mice were treated with 0.684 nmol 5-A-RU/MeG but not 100 nmol 5-A-RU/MeG or CpG (Fig. 3.10 C). Additionally, $CD103^+$ DCs showed a higher percentage of $CCL17^+$ cells in the medLN after stimulation with 0.684 nmol 5-A-RU/MeG, 100 nmol 5-A-RU/MeG and CpG compared to PBS with only PBS to CpG treatment being significant (Fig. 3.10 D). This indicated that MAIT cell activation induced CCL17 expression in DCs.

Besides protein expression levels, expression of CCL17 mRNA was analyzed. With this approach, further chemokines even though the respective reporter mice were not available, could be analyzed. In addition to CCL17, CCL22 was analyzed, which is recognized by the same chemokine receptor as CCL17, CCR4 (Yoshie and Matsushima,

2015). To analyze mRNA expression levels, mice were treated with either PBS, 5-A-RU/MeG or CpG intratracheally followed by isolation of $CD11c^+$ cells 24 h later. As shown in figure 3.11 A, CCL17 mRNA was higher expressed in $CD11c^+$ cells in the lung, while also CCL22 expression was increased in $CD11c^+$ cells of the lung of mice that were treated with 5-A-RU/MeG. Here only the difference between 5-A-RU/MeG and CpG treatment is significant, while the difference between 5-A-RU/MeG and PBS is not significant (Fig. 3.11 B). In the medLN, $CD11c^+$ cells from 5-A-RU/MeG treated mice showed significant higher mRNA expression of CCL17 (Fig. 3.11 C) as well as CCL22 (Fig. 3.11 D) compared to CpG treated mice, while the difference to PBS treated mice is not significant.

This indicated that 5-A-RU/MeG-mediated MAIT cell stimulation caused DCs to produce CCL17 as well as CCL22.

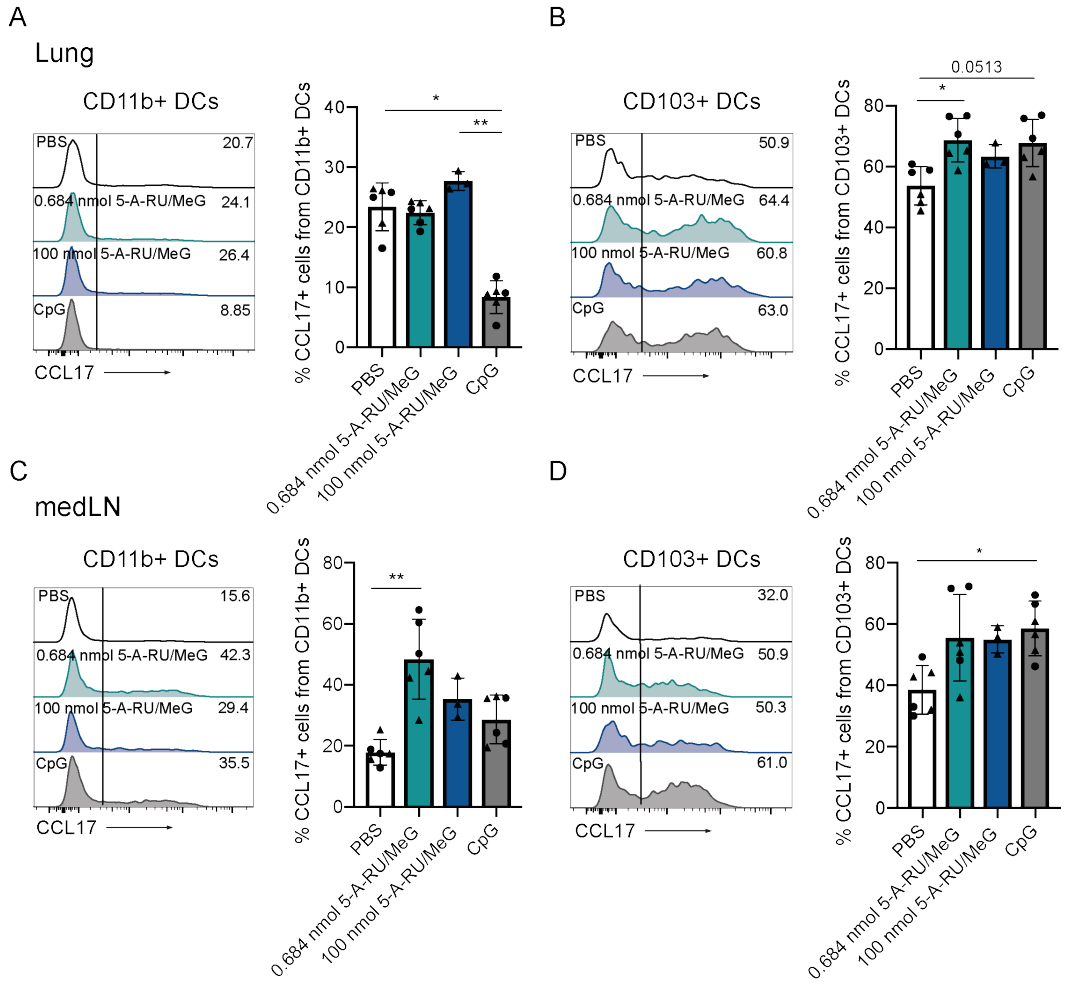


Figure 3. 10 – CCL17 production by DCs in the lung and medLN upon stimulation with either PBS, 5-A-RU/MeG or CpG. (A) Representative histograms of CCL17 in CD11b$^+$ DCs 24 h after application of either PBS, 5-A-RU/MeG or CpG and combined data of the percentage of CCL17$^+$ cells of all CD11b$^+$ DCs in the lung 24 h after treatment with PBS, 5-A-RU/MeG or CpG. (B) Representative histograms of CCL17 in CD103$^+$ DCs 24 h after application of either PBS, 5-A-RU/MeG or CpG and combined data of the percentage of CCL17$^+$ cells of all CD103$^+$ DCs in the lung 24 h after treatment with PBS, 5-A-RU/MeG or CpG. (C) Representative histograms of CCL17 in CD11b$^+$ DCs 24 h after application of either PBS, 5-A-RU/MeG or CpG and combined data of the percentage of CCL17$^+$ cells of all CD11b$^+$ DCs in the medLN 24 h after treatment with PBS, 5-A-RU/MeG or CpG. (D) Representative histograms of CCL17 in CD103$^+$ DCs 24 h after application of either PBS, 5-A-RU/MeG or CpG and combined data of the percentage of CCL17$^+$ cells of all CD103$^+$ DCs in the medLN 24 h after treatment with PBS, 5-A-RU/MeG or CpG. The data represent two independent experiments with 3 mice per group for PBS, 0.684 nmol 5-A-RU/MeG and CpG while one of these experiments was performed with the additional group of 100 nmol 5-A-RU/MeG. The symbol shape represents the different experiments. *P≤0.05, **P≤0.01

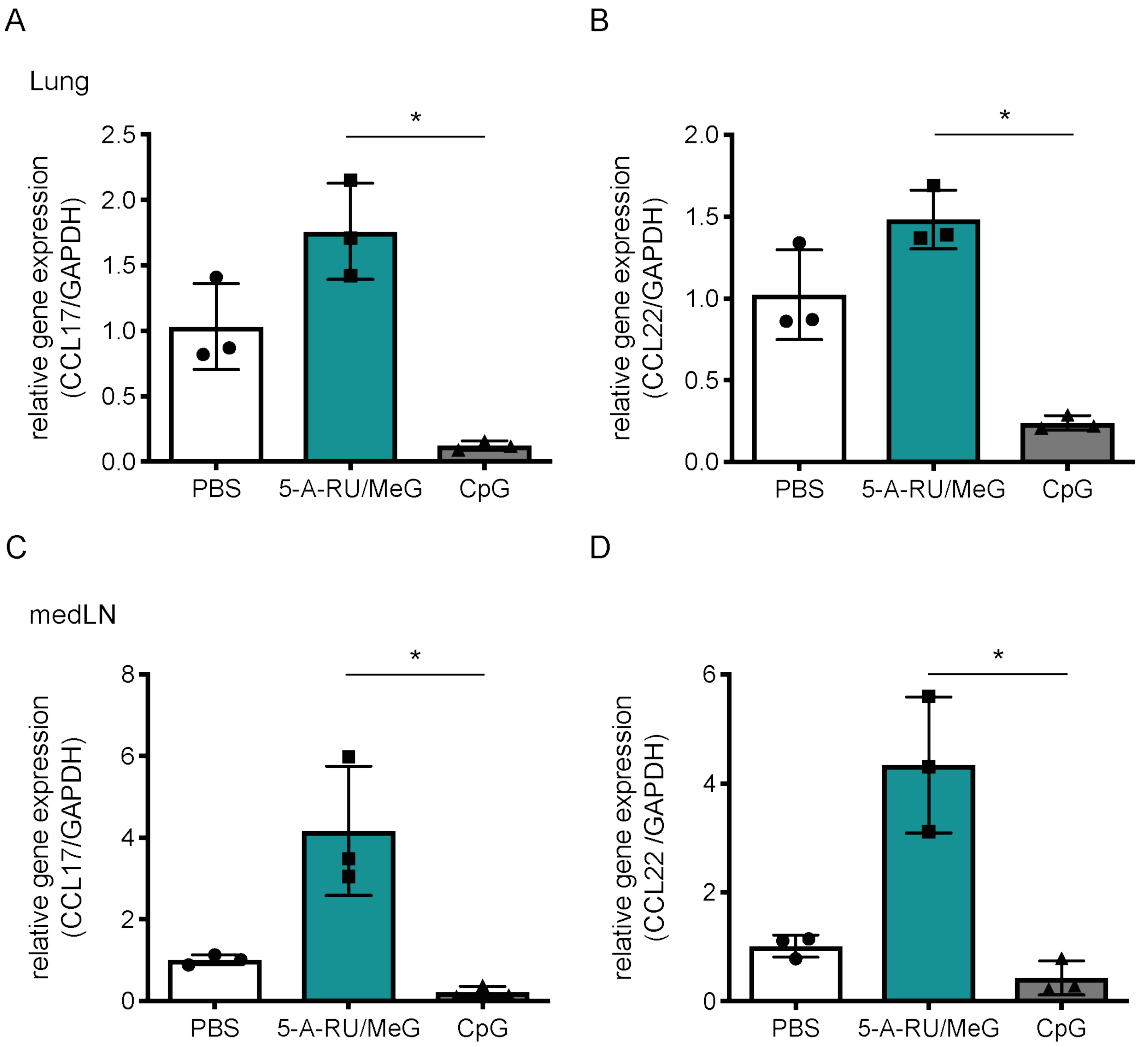


Figure 3. 11 – mRNA expression of CCL17 and CCL22 in $CD11c^+$ cells isolated from the lung and medLN 24 h after intratracheal application of PBS, 0.684 nmol 5-A-RU/MeG or CpG. (A) CCL17 mRNA expression in $CD11c^+$ cells isolated from the lung 24 h after intratracheal application of either PBS, 0.684 nmol 5-A-RU/MeG or CpG. The expression is normalized to GAPDH and PBS treated control. (B) CCL22 mRNA expression in $CD11c^+$ cells isolated from the lung 24 h after intratracheal application of either PBS, 0.684 nmol 5-A-RU/MeG or CpG. The expression is normalized to GAPDH and PBS treated control. (C) CCL17 mRNA expression in $CD11c^+$ cells isolated from the medLN 24 h after intratracheal application of either PBS, 5-A-RU/MeG or CpG. The expression is normalized to GAPDH and PBS treated control. (D) CCL22 mRNA expression in $CD11c^+$ cells isolated from the medLN 24 h after intratracheal application of either PBS, 5-A-RU/MeG or CpG. The expression is normalized to GAPDH and PBS treated control. The data represent one experiment. *P≤0.05

3.2.5. Cytokine production by MAIT cells

Important for the effect of MAIT cells on the immune system is their production of cytokines. Cytokines can modulate the immune response into a more immunogenic or suppressive response, depending on the environment and cytokines that are released. To test, which cytokines MAIT cells produce, MAIT cells were sorted from splenocytes of C57BL/6 mice and cultured with α-CD3 and α-CD28 stimulation supplemented with IL-2. After culture for 4 days, MAIT cells were additionally stimulated with PMA and ionomycin in the presence of GolgiPlug for 4 h to identify the produced cytokines by intracellular staining. As negative control, MAIT cells were stimulated with PMA and ionomycin in the presence of GolgiPlug directly after sorting. As shown in figure 3.12 A, MAIT cells produced IFNγ and IL-17A upon stimulation with PMA and ionomycin without further stimulation. Around 7.6 % of MAIT cells produced IFNγ, while 40 % of MAIT cells are positive for IL-17A, consistent with previous findings (Rahimpour *et al.*, 2015). MAIT cells that were stimulated 4 d with α-CD3 and α-CD28 showed production of IFNγ, IL-17A, GM-CSF, IL-10 and IL-13 after PMA/ionomycin stimulation. 34.7 % of MAIT cells were $IFN\gamma^+$ on d4 of stimulation. IFNγ production was higher in cells that were stimulated for 4 d compared to cells that were not stimulated. In contrast, IL-17A production was similar after α-CD3/ α-CD28 stimulation for 4 d and after no stimulation. On d 4, 38.7 % of MAIT cells were $IL\text{-}17A^+$. Furthermore 36.75 % of them expressed GM-CSF, while 19.7 % expressed IL-10 and 72.2 % expressed IL-13, indicating that murine MAIT cells produce more cytokines than previously thought, especially T_H2 cytokines like IL-13 and regulatory cytokines like IL-10. Besides direct intracellular cytokine staining, cell culture supernatant of MAIT cells that were stimulated with α-CD3 and α-CD28 and IL-2 were collected on day 2 and day 4 of culture. As shown in figure 3.13, a higher amount of IL-13, IL-10, IFNγ,

TNFα, IL-4, GM-CSF, IL-6 and IL-17 was observed on day 4 of cell culture, confirming the results from the intracellular staining (Fig. 3.13).

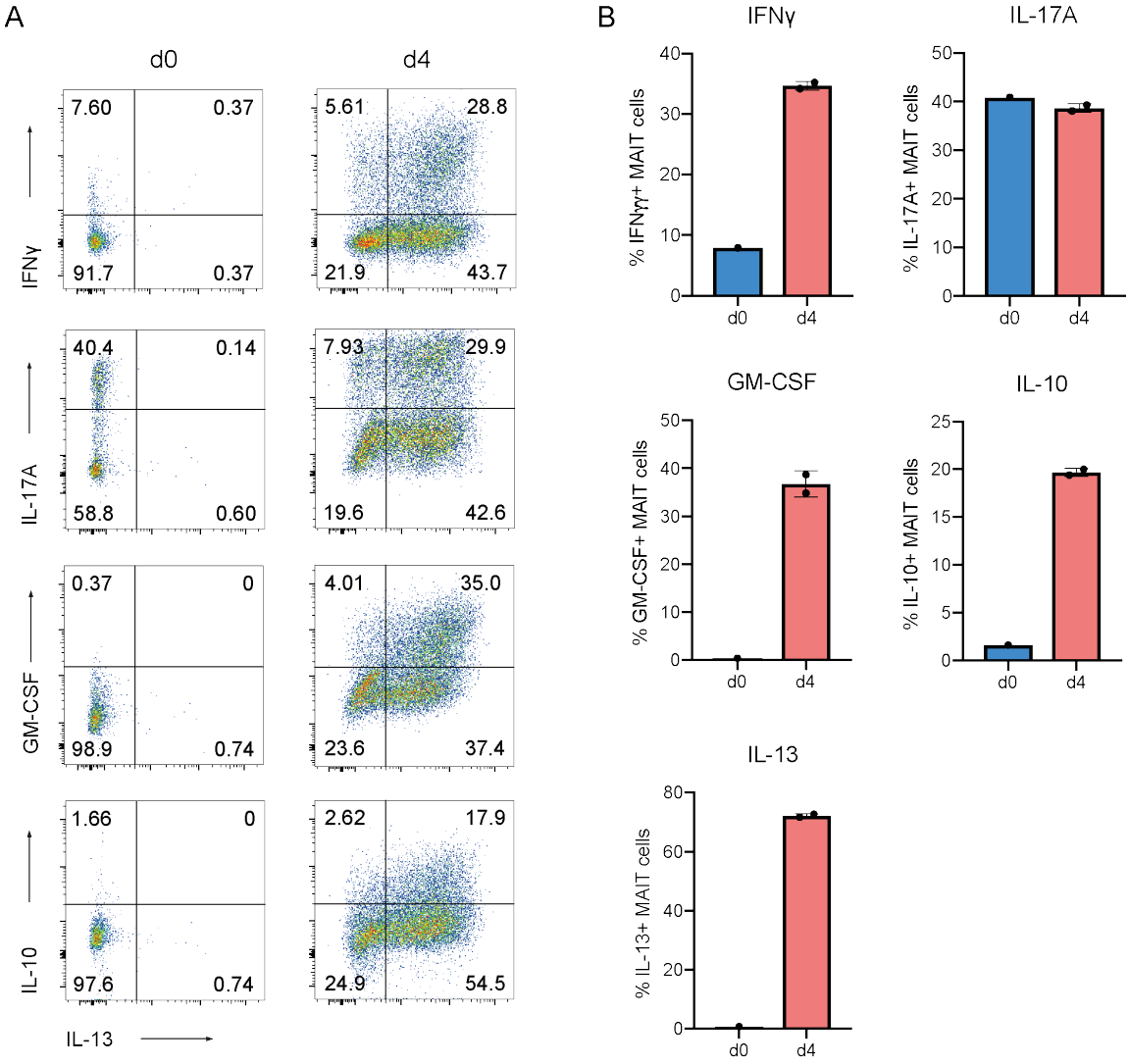


Figure 3. 12 – Intracellular staining of cytokines produced by MAIT cells 4 h after stimulation with PMA/ionomycin in presence of GolgiPlug at day 0 and 4 of culture with α-CD3, α-CD28 and IL-2 stimulation. (A) Representative staining of IFNγ, IL-17A, GM-CSF and IL-10 against IL-13 at day 0 and 4 of culture. (B) Percentages of MAIT cells producing the different cytokines IFNγ, IL-17A, GM-CSF, IL-10 and IL-13. The data represent one experiment.

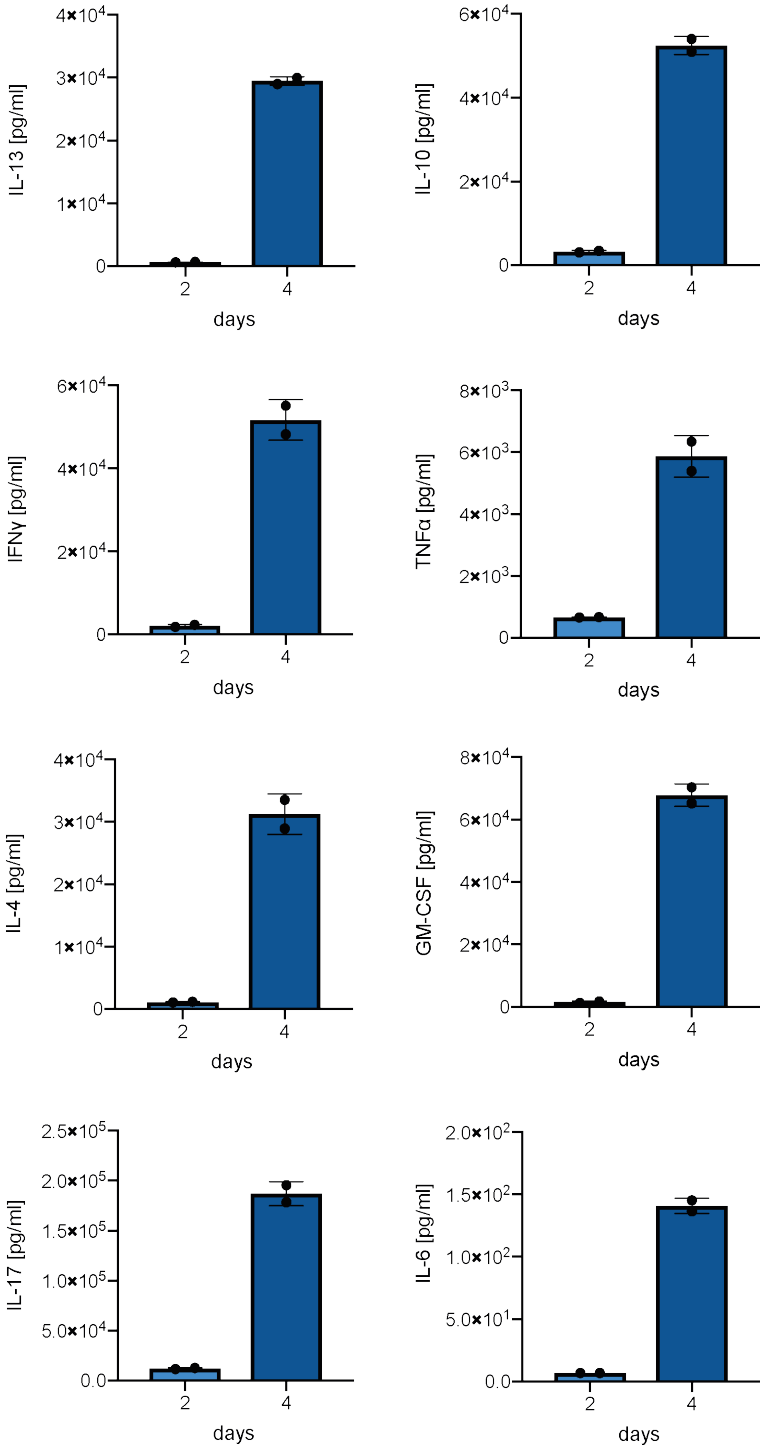


Figure 3. 13 – Cytokine expression of MAIT cells after 2 and 4 days of culture with α-CD3/α-CD28 and IL-2 stimulation. Amount of IL-13, IL-10, IFNγ, TNFα, IL-4, GM-CSF, IL-17 and IL-6 in the supernatant of MAIT cell culture that was stimulated with α-CD3/α-CD28 and IL-2 for 4 days measured by CBA. The data represent one experiment.

3.3. Discussion

Microbial metabolites are able to stimulate MAIT cells *in vivo* and *in vitro.* Most studies used the compound 5-OP-RU. In addition, 5-A-RU can be used for activation of MAIT cells. To this end, 5-A-RU needs to react with MeG to form the activating MAIT cell antigen 5-OP-RU. In this chapter, it was shown that such synthesized 5-A-RU/MeG mixture was able to activate MAIT cells *in vivo*. Intratracheal application of 5-A-RU/MeG activated at least 40 % of MAIT cells. Also *in vitro* studies showed that 5-A-RU generated at the University Bonn could be used to activate MAIT cells in culture. By adding 5-A-RU/MeG to splenocytes, MAIT cells proliferated in a dose-dependent manner. Comparison to 5-OP-RU showed that MAIT cells proliferated less strongly with the same amount of 5-A-RU/MeG. This indicated that 5-A-RU/MeG used here were not as potent as 5-OP-RU in activating MAIT cells, which could be due to an inefficient reaction of 5-A-RU/MeG or to degraded 5-A-RU.

Nevertheless, 5-ARU/MeG was used to analyze the effect of MAIT cell activation on DCs. It was shown that upon MAIT cell stimulation, $CD11b^+$ DCs accumulated within the lung, consistent with a previous study (Meierovics and Cowley, 2016). In that study they showed that during *Francisella tularensis* infection an accumulation of $CD11b^+$ DCs occurred in the lungs of infected mice that resulted from promotion of monocyte differentiation induced by MAIT cells (Meierovics and Cowley, 2016). Analysis of DCs in the medLN showed that higher numbers of both DC subsets were found in the medLN upon stimulation of MAIT cells. Using the PKH26 dye, cells that had migrated from the lung to the medLN could be tracked. Within the medLN, higher numbers of $PKH26^+$ DC subsets were observed, indicating that MAIT cell activation induced migration of DCs from the lung to the medLN.

Furthermore, an upregulation of co-stimulatory molecules was observed on DCs within the lung and medLN after MAIT cell activation, especially by using 100 nmol 5-A-RU/MeG for MAIT cell stimulation. Similar to CpG treated control group mice, MAIT cell stimulation with 100 nmol 5-A-RU/MeG

led to upregulation of CD80, CD86 and CD40 on DCs in the medLN, while 0.684 nmol 5-A-RU/MeG only led to upregulation of CD86 on DCs. Additionally, the expression level of CD86 is lower after 0.684 nmol 5-A-RU/MeG treatment compared to CpG treated mice. This might indicate that MAIT cells can induce partial maturation of DCs when stimulated with a low dose of 5-A-RU/MeG, while high dose 5-A-RU/MeG led to complete maturation of DCs. It was already shown that human MAIT cells could lead to maturation of human DCs *in vitro* (Salio *et al.*, 2017). In this study, all co-stimulatory molecules like CD40, CD80 and CD86 were upregulated, similar to maturation observed after treatment with 100 nmol 5-A-RU/MeG.

Using $MR1^{-/-}$ mice, DC migration and maturation was shown to be MR1 dependent. Comparing $MR1^{-/-}$ mice to C57BL/6J mice, no accumulation of $CD11b^{+}$ DCs was observed in the lungs of $MR1^{-/-}$, which fitted to the published phenotype that MAIT cells promote differentiation of monocytes into DCs in a MR1-depenent manner (Meierovics and Cowley, 2016). Furthermore, similar numbers of $CD103^{+}$ DCs in the lungs of $MR1^{-/-}$ mice were found upon stimulation with either PBS or 5-A-RU/MeG, indicating that the migration of $CD103^{+}$ DCs was impaired. In the medLN, lower or similar numbers of both DC subsets were observed comparing PBS and 5-A-RU/MeG treated mice supporting the hypothesis that MAIT cells induce DC migration. A similar result was obtained when $PKH26^{+}$ DCs were analyzed.

Comparing expression of co-stimulatory molecules between $MR1^{-/-}$ and C57BL/6 mice, clear differences can be observed. In the lung, no upregulation of CD86, CD80 and CD40 expression was observed in $CD11b^{+}$ DCs and $CD103^{+}$ DCs of $MR1^{-/-}$ mice, while upregulation of all markers were observed upon treatment with 100 nmol 5-A-RU/MeG in C57BL/6 mice. This indicated that maturation of DCs in the lung is dependent on MR1. Analyzing DC maturation in the medLN of $MR1^{-/-}$ mice clearly showed no upregulation of CD86, CD80 or CD40 upon stimulation with 5-A-RU/MeG. In contrast C57BL/6J mice showed upregulation of co-stimulatory molecules, especially by injection of 100 nmol 5-A-RU/MeG. This indicated that the observed maturation of DCs in the medLN is MR1-dependent. Furthermore, CpG

treatment in $MR1^{-/-}$ mice showed upregulation of co-stimulatory molecules, indicating that DCs of $MR1^{-/-}$ mice are not impaired in their DC maturation.

Another important finding of this study is the induction of CCL17 expression by MAIT cell activation. This was observed both on protein level and mRNA level. Furthermore, upregulation of CCL22 mRNA expression was observed upon MAIT cell activation. The biological relevance of this finding needs to be established, but it is conceivable that expression of CCL17 and CCL22 might lead to recruitment of $CCR4^+$ T cells, like already reported to occur for NKT cells (Semmling *et al.*, 2010). Furthermore, T_{regs} or T_H2 cells might be recruited, since it has been reported that they express CCR4 as well (Yoshie and Matsushima, 2015).

Analyzing cytokine production by MAIT cells upon TCR stimulation, MAIT cells were able to produce IFNγ and IL-17A as previously reported (Rahimpour *et al.*, 2015). Additionally, they produced high amounts of IL-13 as previously described for human MAIT cells in a study to which I contributed (Kelly *et al.*, 2019). Furthermore, IL-10 production by MAIT cells upon TCR stimulation was observed. IL-13 as well as IL-10 are known to be anti-inflammatory cytokines (De Vries, 1998; Couper *et al.*, 2008).

Furthermore, it was shown that also NKT cells express IL-13 as well as IL-10 (Godfrey and Kronenberg, 2004), showing that MAIT cells produced a cytokine profile similar to NKT cells. Since NKT cell stimulation induced CCL17 and CCL22 expression by DCs, this could explain why a similar upregulation of CCL17 and CCL22 production by DCs was observed upon MAIT cell stimulation.

Altogether, the results presented in this chapter indicated that MAIT cells have an effect on DC migration as well as DC maturation. It needs to be investigated whether this maturation can induce enhanced $CD8^+$ T cell responses like NKT cell activation. This will be addressed in the next chapter. Furthermore, MAIT cells induced CCL17 and CCL22 production by DCs. Whether the observed CCL17 and CCL22 production had a biological relevance like recruiting either $CD8^+$ T cells or $CD4^+$ T cells still is a question that needs further investigation. Nevertheless, a new function of MAIT cells

was uncovered, showing that MAIT cells can have more immunomodulatory capabilities than previously thought.

4. Chapter 4: Effect of MAIT cell on cross-priming

4.1. Introduction

Cytotoxic $CD8^+$ T cells are important components of the adaptive immune response. They recognize peptides loaded onto MHC class I molecules from endogenous viral proteins or cancer cells. In contrast, MHC class II molecules are loaded with peptides from exogenous proteins. The MHC II peptide complex is recognized by $CD4^+$ T cells (Moore *et al.*, 1988; Yewdell *et al.*, 1988). Interestingly, it was shown that also exogenous proteins are able to induce a $CD8^+$ T cell response. The process of loading exogenous peptides on MHC class I is called cross-presentation (Bevan, 1976; Moore *et al.*, 1988; Yewdell *et al.*, 1988; Carbone and Bevan, 1990; Kurts *et al.*, 1996). The mechanism of cross-presented peptides leading to $CD8^+$ T cell responses is called cross-priming (Bevan, 1976). It was shown that DCs can cross-present antigens more efficiently than other immune cells and are able to induce T cell responses by cross-priming (Den Haan *et al.*, 2000b; del Rio *et al.*, 2007). For induction of a T cell response, DCs take up antigen and undergo maturation and migrate to the lymph node, where the antigen is presented to T cells (Savina and Amigorena, 2007). Three different signals are postulated to induce a $CD8^+$ T cell response. The first signal is the recognition of peptide presented via MCH class I, the second signal is upregulation of co-stimulatory molecules on DCs (Bretscher and Cohn, 1970) and the third signal is the production of cytokines by DCs (Curtsinger *et al.*, 1999). Besides co-stimulatory molecules, the help of $CD4^+$ T cells is required to activate DCs, so that they can induce an efficient $CD8^+$ T cell response (Bennett *et al.*, 1997; Hamilton-Williams *et al.*, 2005). The help provided by $CD4^+$ T cells is mainly facilitated by interaction of CD40L on $CD4^+$ T cells and CD40 on DCs (Carbone *et al.*, 1998; Bennett *et al.*, 1998). Besides $CD4^+$ T cells, it was shown that NKT cells can enhance $CD8^+$ T cell responses by providing help for DCs (Stober *et al.*, 2003; Ian F Hermans *et al.*, 2003; Semmling *et al.*, 2010).

By injection of α-galactosylceramide (αGalCer), the glycolipid that is presented via CD1d, NKT cells were activated, which in turn led to maturation of DCs, which was measured by upregulation of CD80 and CD86 (Fujii *et al.*, 2003; Ian F Hermans *et al.*, 2003). Furthermore, it was shown that the help of NKT cells was facilitated by CD40L on NKT cells (Ian F Hermans *et al.*, 2003). Additionally, NKT cells induced IL-12 production by DCs (Tomura *et al.*, 1999).

Besides the postulated three signals for inducing efficient $CD8^+$ T cell response, it was shown that chemokines, that recruit specific immune cells, can act as signal 0 (Bousso and Albert, 2010). NKT cells promote production of CCL17 by DCs, which leads to recruitment of $CCR4^+$ $CD8^+$ T cells. This in turn enhanced the likelihood of $CD8^+$ T cells to encounter licensed DCs (Semmling *et al.*, 2010). In contrast, DCs produce CCL3, CCL4 and CCL5 upon $CD4^+$ T cell help, which leads to recruitment of $CCR5^+$ $CD8^+$ T cells (Castellino *et al.*, 2006).

Since NKT cells and MAIT cells share many features, like invariant TCR as well as rapid cytokine production upon activation, it is hypothesized that also MAIT cells are able to enhance $CD8^+$ T cell priming. Due to DC maturation observed in chapter 3, MAIT cells may have the ability to enhance cross-priming by augmenting DC maturation as well as by induction of CCL17 production, which then might lead to recruitment of $CCR4^+$ $CD8^+$ T cells. To test the hypothesis that MAIT cell can enhance cross-priming, the model antigen system ovalbumin (OVA) was used. By transferring OVA specific $CD8^+$ T cells (OT-I cells) prior to vaccination with OVA and MAIT cell antigen, the efficiency of vaccination by analyzing the OT-I cell numbers as well as determining the cytotoxic capacity of OT-I cells after vaccination could be tracked. The aim of this chapter was therefore to analyze the effect of MAIT cell activation on the $CD8^+$ T cell response in a vaccination system.

4.2. Results

4.2.1. The effect of MAIT cell activation on cross-priming

The previous results showed that MAIT cell activation could induce DC migration and maturation of $CD103^+$ DCs. To clarify whether the found DC maturation by MAIT cell activation could enhance cross-priming of $CD8^+$ T cells, OVA-specific $CD8^+$ T cells (OT-I cells) were transferred prior to vaccination. Mice were vaccinated intratracheally with different combinations of 5-A-RU/MeG and CpG together with OVA. 6 days (d) after vaccination, an *in vivo* cytotoxic assay was performed to determine the cytotoxic ability of antigen-specific $CD8^+$ T cells. To this end, target cells, pulsed with the OVA peptide SIINFEKL and unpulsed, were transferred by intravenous injection. 4h after transfer of the target cells the frequency of the SIINFEKL pulsed cells and unpulsed target cells was determined to calculate the OVA-specific cytotoxic activity, since OT-I cells should only recognize and eliminate the cells loaded with SIINFEKL. The cytotoxic efficiency and OT-I cell numbers were determined in the lung, medLN as well as in the spleen.

No enhanced cytotoxicity was observed after immunization with 5-A-RU/MeG + OVA in the lung (Fig. 4.1 A). In contrast, vaccination with CpG + OVA showed 30 % cytotoxic activity. The cytotoxicity resulting from CpG vaccination was not altered by addition of 5-A-RU/MeG (Fig. 4.1 A). Analyzing the numbers of OT-I cells, it was shown that only in groups that received CpG + OVA w/o 5-A-RU/MeG OT-I cells were significantly more frequent compared to groups that were not vaccinated with CpG. In the groups that received 5-A-RU/MeG + OVA or OVA alone, lower OT-I cell numbers were found compared to the two groups that had received CpG (Fig. 4.1. B). Analyzing the numbers of MAIT cells, the group that received 5-A-RU/MeG + CpG + OVA showed significant higher number of MAIT cells compared to the other groups, consistent with previous studies showed that MAIT cell proliferation only occurred if antigen was applied together with a TLR ligand (Chen *et al.*,

2016). Significant higher MAIT cell numbers were found in the lungs after injection of 5-A-RU/MeG together with TLR9 agonist CpG compared to all other groups (Fig. 4.1 C). This indicated that the MAIT cell stimulation and vaccination technique used here worked in mice. Even though MAIT cells recognized their antigen and were activated by intratracheal injection of 5-A-RU/MeG, no enhancement of $CD8^+$ T cell response was observed in the lung, as measured by cytotoxicity and OT-I cell numbers. In the medLN, vaccination showed results similar to those observed in the lungs. Only the groups that received CpG showed a greater cytotoxic activity, while 5-A-RU/MeG did not enhance the cytotoxic activity. Furthermore, injection of 5-A-RU/MeG with OVA induced a cytotoxic activity of only 12 % (Fig.4.2 A). Also the OT-I cell numbers were increased only when CpG was injected (Fig.4.2 B). Interestingly, there was a tendency that mice with full vaccination (5-A-RU/MeG + CpG + OVA) showed the highest OT-I cell numbers. In contrast, immunization with 5-A-RU/MeG + OVA showed similar OT-I cell numbers to immunization with OVA alone (Fig. 4.2 B). Analyzing the MAIT cell numbers in the lymph node, only the group with full vaccination (5-A-RU/MeG + CpG + OVA) showed increased MAIT cell numbers similar to the results obtained from the lung (Fig 4.2 C). Comparing MAIT cell numbers between medLN and lung, lower MAIT cell numbers were found in the medLN compared to the lung, which might be an effect of lower MAIT cell numbers in the lymph node in general. These results indicated that also in the medLN, MAIT cell activation did not enhance cytotoxicity of OT-I cells as well as increased OT-I cell numbers.

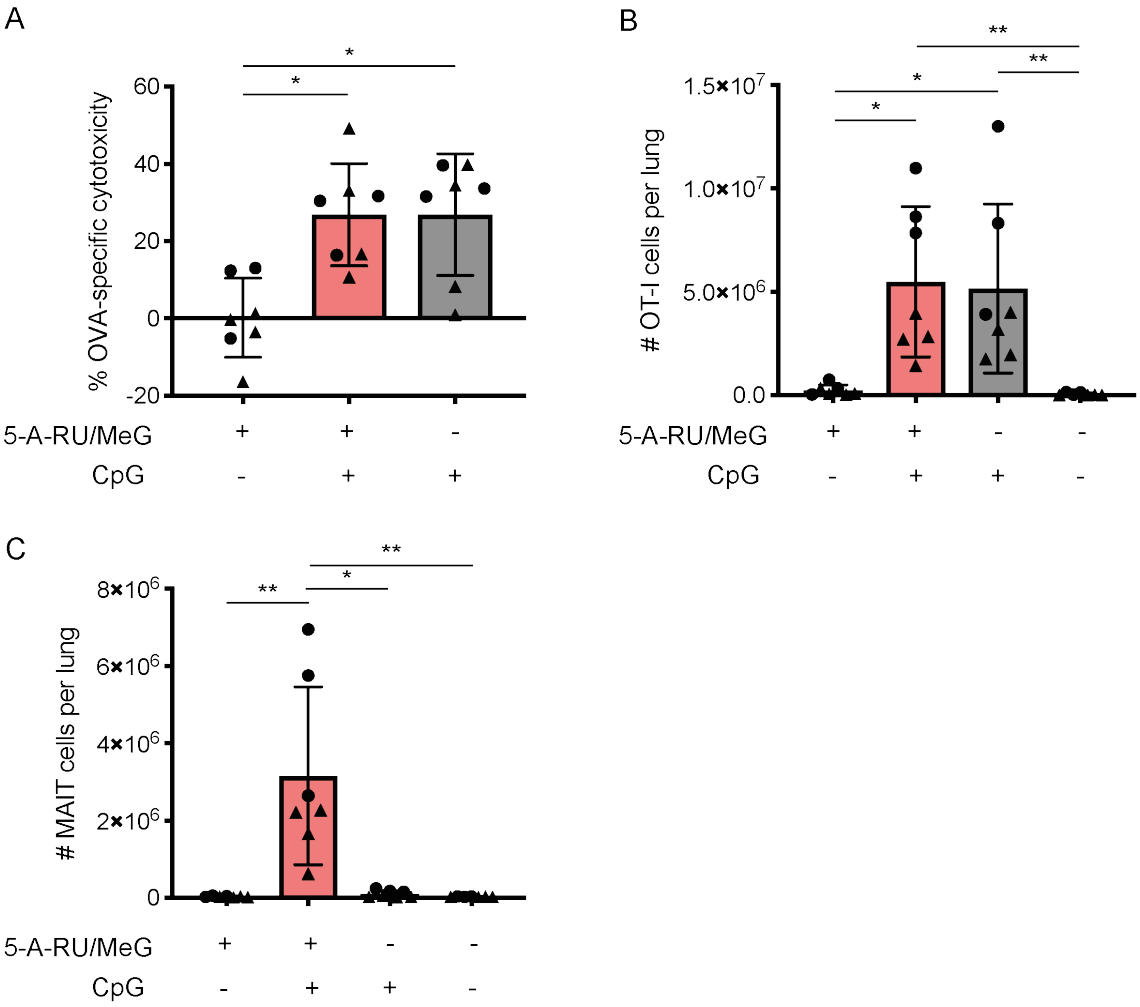


Figure 4. 1 - Cross-priming of $CD8^+$ T cells after MAIT cell stimulation in the lung. OT-I cells were transferred prior to vaccination. The mice were vaccinated by intratracheal administration using 5-A-RU/MeG + OVA, 5-A-RU/MeG + CpG + OVA, CpG + OVA and OVA alone. 6 d later SIINFEKL pulsed and unpulsed target cells were transferred to analyze the specific kill of SIINFEKL pulsed target cells by OT-I cells 4 h after target cell injection. (A) *In vivo* OVA-specific cytotoxicity in the lung 6 d after vaccination. (B) OT-I cell numbers 6 d after vaccination in the lung. (C) MAIT cell numbers per lung of mice 6 d after vaccination. 6-7 mice per group from 2 independent experiments. The dots and triangles represent the different experiments. In the experiment represented by dots also antigen-specific $CD4^+$ T cells were transferred. *P≤0.05, **P≤0.001 using Kruskal-Wallis test.

Besides the lung and medLN, also the spleens were analyzed for cytotoxic activity of OT-I cells as well as the OT-I cell numbers, because this organ is known to be the major organ for cross-priming besides the

lymph nodes. A cytotoxic activity of 75% was obtained when mice were fully vaccinated (5-A-RU/MeG + CpG + OVA), while 89% cytotoxic activity were observed when mice were vaccinated with CpG + OVA, indicating that 5-A-RU/MeG did not enhance $CD8^+$ T cell priming and cytotoxicity. 5-A-RU/MeG + OVA did not lead to an efficient priming of $CD8^+$ T cells, since only a cytotoxic efficiency of 14% was observed (Fig. 4.3 A). For OT-I cell numbers, only the groups that received CpG showed a not significant expansion of OT-I cells compared to the group that had just received OVA. Also here, the addition of 5-A-RU/MeG did not enhance accumulation of OT-I cells compared to full-vaccinated mice. Furthermore, 5-A-RU/MeG + OVA did not enhance expansion of OT-I cells (Fig 4.3 B). However, an expansion of MAIT cell numbers was observed in the group that was vaccinated using 5-A-RU/MeG + CpG + OVA although the MAIT cell number was not significantly increased to the other groups (Fig 4.3 C). The tendency of higher MAIT cell numbers after vaccination with 5-A-RU/MeG + CpG + OVA reflects the results already observed in the lung and medLN (Fig. 4.1 C and Fig. 4.2 C). Taken together, these results failed to support the hypothesis that MAIT cell activation can enhance the cytotoxic activity or proliferation of $CD8^+$ T cells in the spleen.

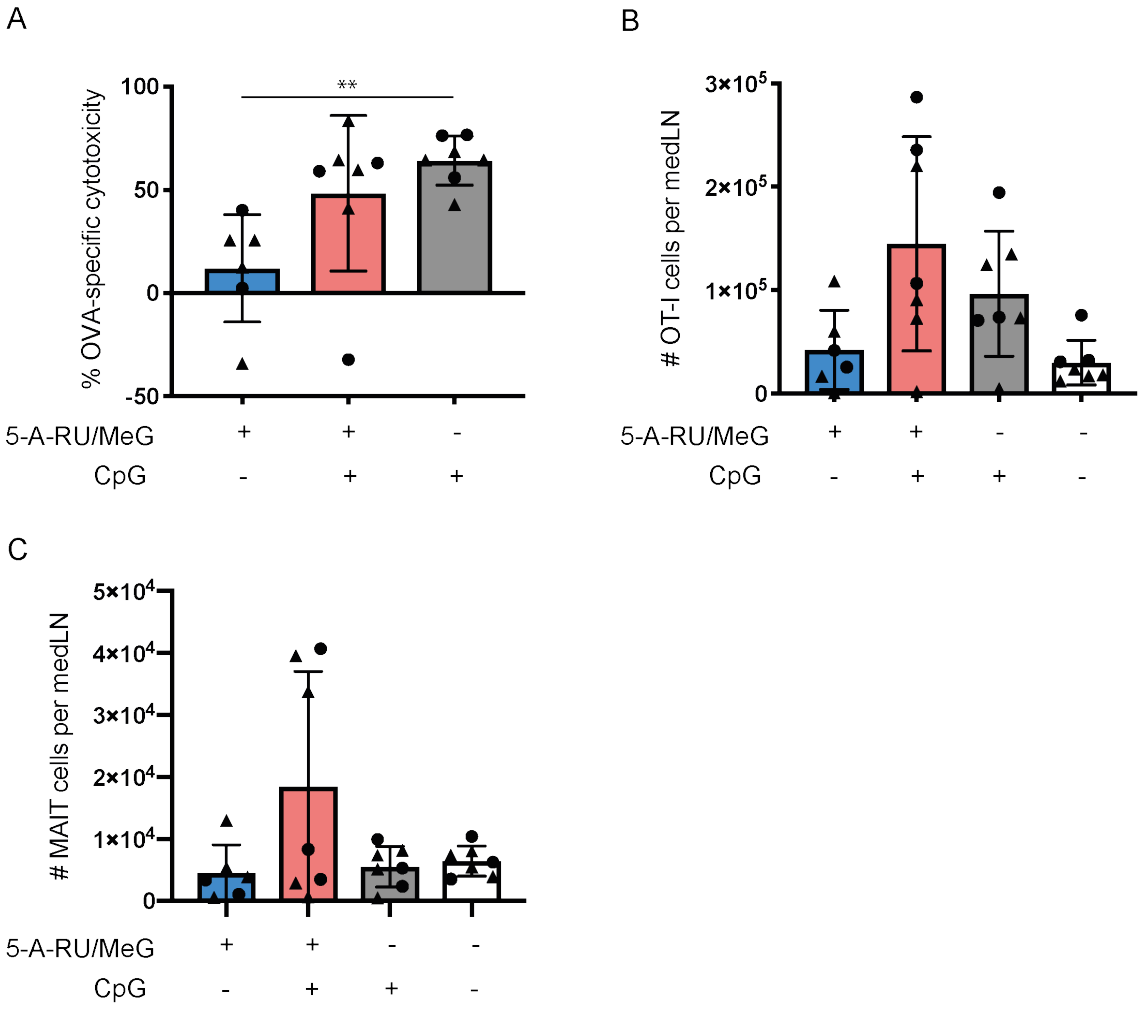


Figure 4. 2 - Cross-priming of $CD8^+$ T cells in the medLN after intratracheal MAIT cell stimulation. OT-I cells (CD45.1) were transferred prior to vaccination. The mice were vaccinated by intratracheal administration using 5-A-RU/MeG + OVA, 5-A-RU/MeG + CpG + OVA, CpG + OVA and OVA alone. 6 d later SIINFEKL pulsed and unpulsed target cells were transferred to analyze the specific kill of SIINFEKL pulsed target cells by OT-I cells 4 h after target cell injection. (A) *In vivo* OVA-specific cytotoxicity in the medLN 6 d after vaccination. (B) OT-I cell numbers in the medLN of vaccinated mice after 6 d. (C) MAIT cell numbers per medLN of mice 6 d after vaccination. 6-7 mice per group from 2 independent experiments. In the experiment represented by dots also antigen-specific $CD4^+$ T cells were transferred. The dots and triangles represent the different experiments. **P≤0.001 using Kruskal-Wallis test.

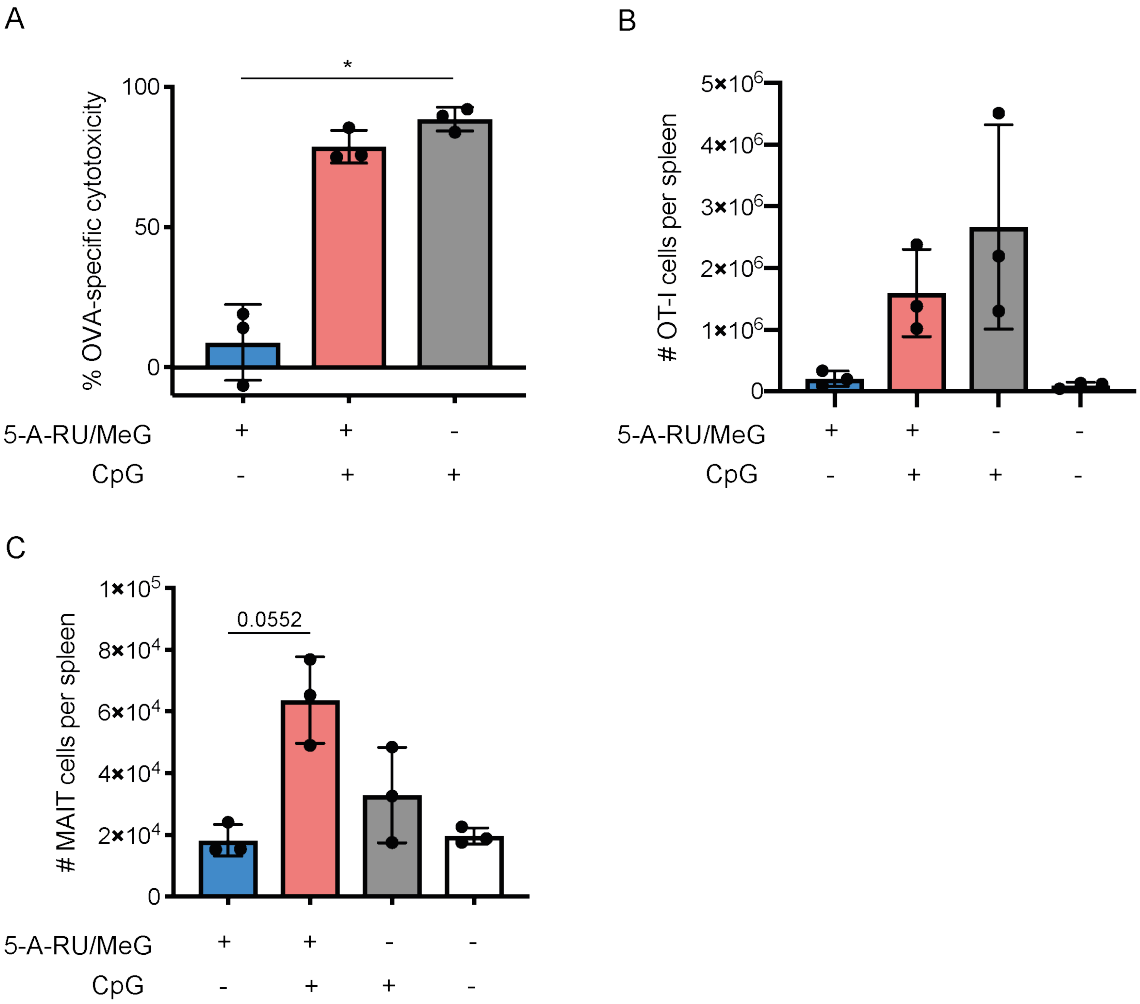


Figure 4. 3 - Cross-priming of $CD8^+$ T cells in the spleen after intratracheal MAIT cell stimulation. OT-I cells (CD45.1) were transferred prior to vaccination. The mice were vaccinated by intratracheal administration using 5-A-RU/MeG + OVA, 5-A-RU/MeG + CpG + OVA, CpG + OVA and OVA alone. 6 d later SIINFEKL pulsed and unpulsed target cells were transferred to analyze the specific kill of SIINFEKL pulsed target cells by OT-I cells 4 h after target cell injection. (A) *In vivo* OVA-specific cytotoxicity in the spleen 6 d after intratracheal vaccination. (B) OT-I cell numbers 6 d after vaccination in the spleen. (C) MAIT cell numbers per spleen 6 d after vaccination. Data show one experiment with 3 mice per group. *P≤0.05 using Kruskal-Wallis test.

In addition to the antigen-specific response from antigen-specific $CD8^+$ T cells, the response of the endogenous $CD19^+$ B cells as well as of endogenous $CD8^+$ and $CD4^+$ T cells were analyzed. As shown in figure 4.4 A, no significant differences were observed in the numbers of $CD19^+$ cells in the lung, medLN and spleen in response to the different

vaccinations. Nevertheless, a tendency of higher $CD19^+$ cell numbers after vaccination with 5-A-RU/MeG was observed in the lung and medLN, but the high variance within each group did not enable clear conclusions (Fig. 4.4 A). Likewise, no significant differences were observed in the numbers of $CD8^+$ cells in the lung and medLN. Vaccination with 5-A-RU/MeG + CpG + OVA or CpG + OVA showed a tendency of higher $CD8^+$ cells numbers compared to the other groups that did not received CpG in the lung and medLN (Fig. 4.4 B). In the spleen the highest numbers of $CD8^+$ cells was found in mice that were immunized with CpG + OVA. Here the result was significant compared to mice that received OVA alone (Fig. 4.4 B). The other vaccinations did not lead to a higher number of $CD8^+$ cells (Fig. 4.4 B). As shown in figure 4.4 C, the highest numbers of $CD4^+$ cells were found within in the lungs and medLN of mice that received either 5-A-RU/MeG + CpG + OVA or CpG + OVA as vaccination. However, the results here were not significant due to high variance within each group. In the spleen no differences in $CD4^+$ cell numbers were observed (Fig. 4.4 C). These results suggested that MAIT cell activation did not change the numbers of endogenous $CD19^+$, $CD8^+$ or $CD4^+$ T cells.

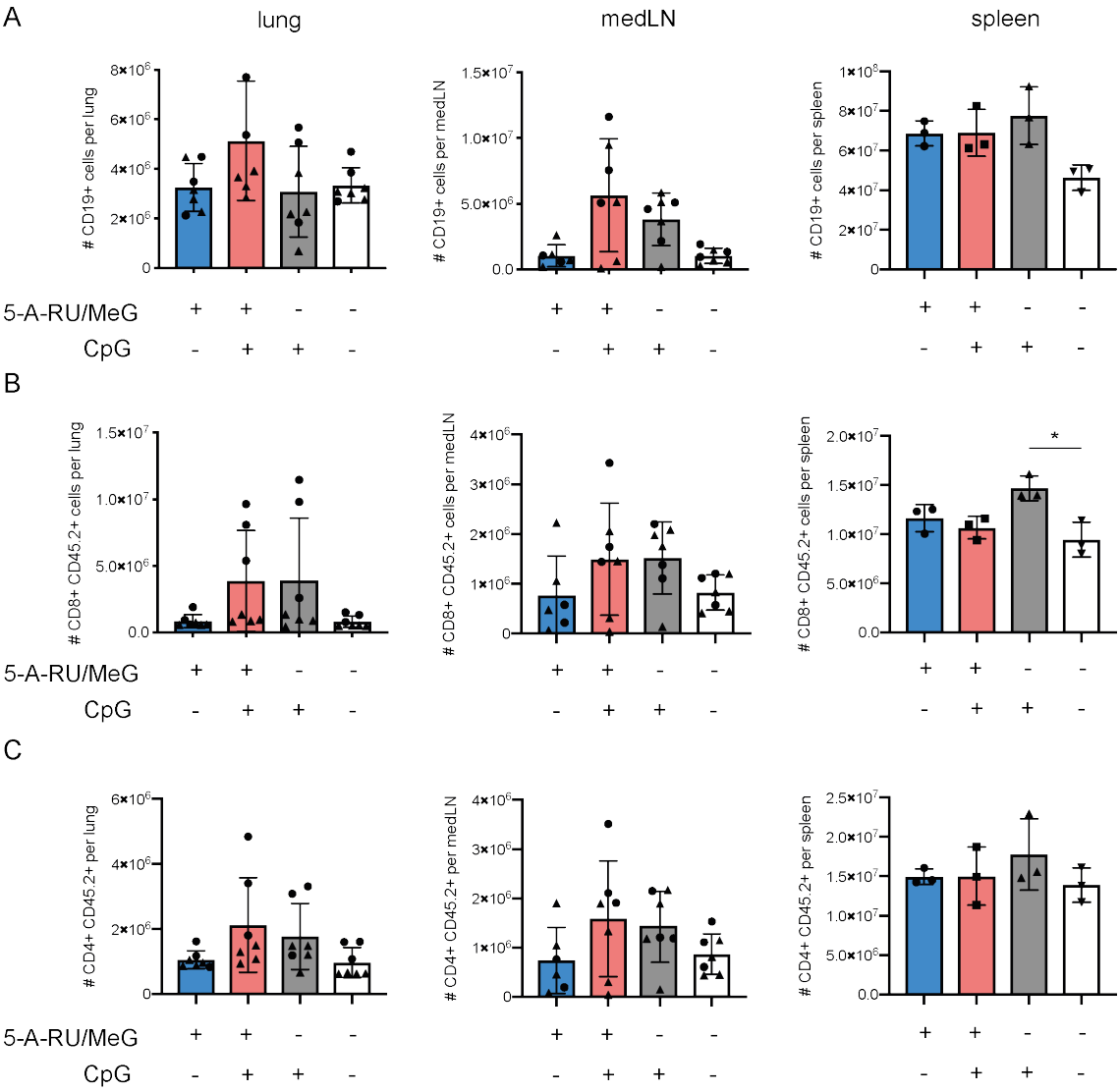


Figure 4. 4 – Cell numbers of CD19$^+$, CD8$^+$ and CD4$^+$ cells 6 d after vaccination and *in vivo* cytotoxic assay. (A) CD19$^+$ cells in the lung, medLN and spleen 6 d after vaccination with 5-A-RU/MeG + OVA, 5-A-RU/MeG CpG + OVA, CpG + OVA or OVA and *in vivo* cytotoxic assay. (B) CD8$^+$ CD45.2$^+$ cells in the lung, medLN and spleen 6 d after vaccination with 5-A-RU/MeG + OVA, 5-A-RU/MeG CpG + OVA, CpG + OVA or OVA and *in vivo* cytotoxic assay. (C) CD4$^+$ CD45.2$^+$ cells in the lung, medLN and spleen 6 d after vaccination with 5-A-RU/MeG + OVA, 5-A-RU/MeG CpG + OVA, CpG + OVA or OVA and *in vivo* cytotoxic assay. The data from the lung and medLN are from 2 independent experiments. Round dots and triangles indicate the different experiments. In the experiment represented by dots antigen-specific CD4$^+$ T cells were injected together with OT-I cells. The data from the spleen is from one experiment. *P≤0.05 using Kruskal-Wallis test.

All together, these results suggested that MAIT cell activation did not lead to enhancement of antigen-specific $CD8^+$ T cell responses as well as higher numbers of endogenous $CD19^+$, $CD8^+$ or $CD4^+$ cells. One explanation could be that the vaccination was applied intratracheally, which might be not efficient enough to achieve a $CD8^+$ T cell response. Therefore, an experiment was performed, where mice were vaccinated by intravenous injection. All components were injected in higher doses due to intravenous injection. For 5-A-RU/MeG 50 nmol was injected, while 20 µg CpG and 200 µg OVA were used. 5 d after vaccination, the $CD8^+$ T cell response to vaccination was analyzed using an *in vivo* cytotoxic assay as described previously. A cytotoxic activity of 60 % was found in the spleen after vaccination with CpG, no matter if 5-A-RU/MeG was co-administered. Injection of 5-A-RU/MeG + OVA led to a cytotoxic activity of 20 % compared to vaccination with OVA alone (Fig. 4.5 A). Analyzing the OT-I cell numbers, groups, which received CpG, showed higher OT-I cell numbers compared to OVA vaccinated mice (Fig. 4.5 B). Interestingly, the MFI of CD44 on OT-I cells was lower in groups, where CpG was injected while the group, which received 5-A-RU/MeG + OVA showed the highest MFI of CD44 (Fig. 4.5 C). To prove that our injection of 5-A-RU/MeG led to activation of MAIT cells, the numbers of MAIT cells were analyzed. As shown in figure 4.5 D, the highest number of MAIT cells was found in the group that received 5-A-RU/MeG + CpG as well as OVA, which was expected, as administration of MAIT cell antigen together with a TLR stimulation result in proliferation of MAIT cells. Interestingly, mice, which received CpG + OVA or 5-A-RU/MeG + OVA also, showed a tendency towards higher number of MAIT cells compared to control treated mice that received OVA alone. However, the differences in MAIT cell numbers were not statistically significant (Fig. 4.5 D).

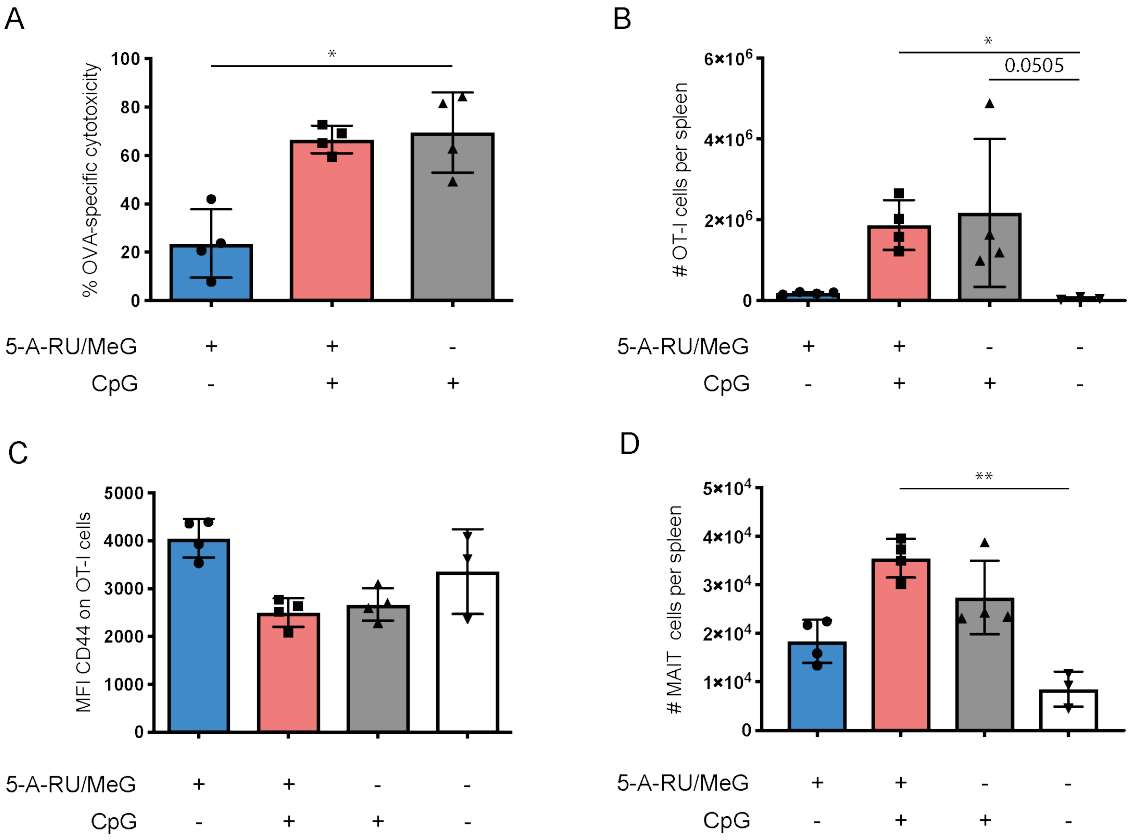


Figure 4.5 – MAIT cell activation did not enhance cross-priming using a intravenous vaccination route. OT-I cells were transferred into mice followed by vaccination with 5-A-RU/MeG + OVA w/o CpG as well as CpG + OVA or OVA alone on the next day. 5 d after vaccination target cells w/o loaded SIINFEKL were transferred to analyze the specific kill of the SIINFEKL pulsed cells after 4 h. (A) *In vivo* OVA-specific cytotoxic response in spleen 5 d after vaccination. (B) Numbers of OT-I cells per spleen 5 d after vaccination. (C) Mean fluorescence intensity of CD44 on OT-I cells in the spleen 5 d after vaccination. (D) Numbers of MAIT cells per spleen 5 d after vaccination. The data represent one experiment.

4.3. Discussion

In this section, the hypothesis that MAIT cells can enhance $CD8^+$ T cell priming was investigated. By using the model antigen system OVA, antigen-specific T cells as well as vaccination efficiency was tracked. By injection of OVA-specific $CD8^+$ T cells (OT-I cells) and vaccination, it was shown that MAIT cell activation did not enhance $CD8^+$ T cell priming and did not increase cytotoxicity of $CD8^+$ T cells. No matter if 5-

A-RU/MeG was injected with OVA or together with OVA and CpG, no enhancement of $CD8^+$ T cell response was observed in the lung, medLN or the spleen compared to mice that were vaccinated with OVA and CpG. Furthermore, no difference in OT-I cell numbers were observed by MAIT cell activation.

As described in chapter 3, only upregulation of CD86 upon activation of MAIT cells was found when 0.684 nmol 5-A-RU/MeG was injected, while CD80 and CD40 were not upregulated on DCs by 0.684 nmol 5-A-RU/MeG. CD40 plays an important role in inducing efficient $CD8^+$ T cell response, since $CD4^+$ T cell and NKT cell help is provided trough the CD40-CD40L axis (Bennett *et al.*, 1998; Ian F. Hermans *et al.*, 2003). The lack of CD40 on DCs in this experimental system might explain why no enhanced $CD8^+$ T cell response was observed since an integral signal for DC activation is missing. Here only the dose of 0.684 nmol 5-A-RU/MeG was tested for vaccinations and additional experiments with 100 nmol 5-A-RU/MeG are necessary, since it was shown in chapter 3 that this dose lead to full maturation of DCs. Therefore vaccination experiments using 100 nmol 5-A-RU/MeG are indispensable.

Additionally, a different route of vaccination was used to exclude the potential lack of efficient $CD8^+$ T cell response by the chosen route of vaccination. By using also intravenously vaccination, no enhanced $CD8^+$ T cell priming was observed, indicating that the route of vaccination is not responsible for the lack of efficient $CD8^+$ T cell response. However, also here a lower dose than 100 nmol 5-A-RU/MeG was used for intravenously vaccination, which could lead to inefficient $CD8^+$ T cell response. Therefore, this experiment needs to be repeated with additional higher dose of 5-A-RU/MeG.

Furthermore, the effect of MAIT cells might be analyzed using suboptimal doses of CpG. Suboptimal doses of CpG could lead to MAIT cell proliferation, but not to a $CD8^+$ T cell response by CpG injection itself. By additional TLR stimulation, the MAIT cell activation might boost

$CD8^+$ T cell response without inducing a CpG driven $CD8^+$ T cell response.

Besides antigen-specific $CD8^+$ T cell responses, the endogenous $CD19^+$, $CD8^+$ and $CD4^+$ cell responses were analyzed. CD19 was used to analyze B cells, but no differences in B cell numbers, nor in $CD8^+$ and $CD4^+$ cell numbers were observed. Since no antigen-specific responses were analyzed, the possibility that there was an enhanced response of antigen-specific cells could not be excluded. To test this, further investigations need to be done.

In chapter 3, it was shown that MAIT cells were able to produce suppressive cytokines upon activation *in vitro*. Furthermore, an incomplete maturation of DCs was observed with the dose of 5-A-RU/MeG used for vaccinations. Using a dose of 0.684 nmol 5-A-RU/MeG the co-stimulatory molecules CD40 and CD80 are not upregulated *in vivo*. These results, together with no enhancement of $CD8^+$ T cell response, might indicate that MAIT cells induce a more tolerogenic immune response when 5-A-RU/MeG is injected in low dose (here 0.684 nmol 5-A-RU/MeG).

Also differentiation of $CD4^+$ T cells could be affected by MAIT cell activation. Thus, it was shown that *Staphylococcus aureus* PSM peptides could induce tolerogenic DCs by downregulation of CD40 on DCs that were activated with different TLR ligands. Those DCs were able to induce regulatory T cells (Armbruster *et al.*, 2016). Maybe also MAIT cells induce regulatory T cells by activation of tolerogenic DCs and production of suppressive cytokines. To test this hypothesis, dedicated experiments need to be done. On the one hand, the cytokines produced by DCs would need to be analyzed, while on the other hand the numbers of regulatory T cells should be analyzed upon stimulation of MAIT cells. Additionally, other doses of 5-A-RU/MeG need to be analyzed for vaccination strategies,

Furthermore, the cytokines produced by MAIT cells *in vivo* should be determined, since the results were obtained from *in vitro* stimulation so

far, which can differ to the actual cytokines produced *in vivo.* It is possible that MAIT cells did not show suppressive cytokine production *in vivo.*

Taken together, the results in this chapter demonstrate that MAIT cells do not have the ability to enhance $CD8^+$ T cell responses, at least in the experimental setup used. Nevertheless, it opens the question of a functional role of MAIT cells within the immune system and whether they possess a more suppressive function on other immune cells. Further studies need to be executed to clarify the role of MAIT cells on conventional $CD8^+$ or $CD4^+$ T cells and if MAIT cells can be harnessed to treat inflammatory diseases.

5. Chapter 5: FOXP3$^+$ T-bet$^+$ MR1-5-OP-RU$^+$ T cells in human thymus

5.1. Introduction

Human MAIT cells express the invariant TCR α-chain TRAV1-2 (Vα7.2) TRAJ33 (Tilloy *et al.*, 1999) and can be identified using MR1-5-OP-RU tetramers (Rahimpour *et al.*, 2015). Before establishment of MR1 tetramers, MAIT cells were identified using surrogate markers like Vα7.2 and CD161. This method of identification is not optimal since other T cells can express Vα7.2 as well as CD161. Besides MAIT cells, recent studies identified cells that could bind to MR1 tetramers but do not express Vα7.2. These MR1 tetramer$^+$ TRAV1-2$^-$ cells are reported to also bind MR1-6-FP tetramers (Gherardin *et al.*, 2016; Koay *et al.*, 2019). MR1-6-FP tetramers are normally used as negative control staining for MR1-5-OP-RU tetramers in MAIT cells. Further analysis of MR1 tetramer$^+$ TRAV1-2$^-$ cells showed that some of them expressed typical MAIT cells markers like CD161, IL-18R (CD218), CD26 and PLZF (promyelocytic leukemia zinc finger, Zbtb16), a transcription factor that is known to be involved in MAIT cell and NKT cell development (Savage *et al.*, 2008; Koay *et al.*, 2016). They are called non-classical MAIT cells. In contrast, some MR1 tetramer$^+$ TRAV1-2$^-$ cells lack these markers and are referred to as atypical MR1-resticted T cells. The cells that lack the expression of the typical MAIT cell markers CD161, IL-18R, CD26 and PLZF could be stained with MR1-6-FP tetramers (Koay *et al.*, 2019). Since their appearance is different and since they do not express the transcription factor PLZF that is involved in MAIT cell development, it is suggested that atypical MR1-restricted T cells have a distinct developmental pathway compared to MAIT cells. Besides PLZF, atypical MR1-restricted T cells differently express other transcription factors compared to MAIT cells. MAIT cells normally express T-bet as well as RoRγT, while atypical MR1-restricted MAIT cells are negative for RoRγT

and have a heterogeneous expression of T-bet (Gherardin *et al.*, 2016).

MAIT cells undergo positive selection within the thymus (Tilloy *et al.*, 1999; Martin *et al.*, 2009). It is believed that MAIT cells randomly rearrange their TCR and are selected into MAIT cell linage if the TCR is able to interact with $CD4^+CD8^+$ thymocytes that express MR1 (Tilloy *et al.*, 1999; Martin *et al.*, 2009; Seach *et al.*, 2013), while conventional T cells needs interaction of TCR with MHC molecules on thymic epithelial cells during positive selection (Murphy *et al.*, 2012). During MAIT cell development three different developmental stages can be distinguished. The surface markers CD27 and CD161 were used to identify these stages within human thymus. Furthermore, the stages differ in expression of CD8 and CD4. Stage 1 cells are mainly $CD4^+CD8^+$, but also some $CD4^+CD8^-$ and $CD4^-CD8^+$ were observed within this stage. In stage 2, CD8 and CD4 expression was similar to stage 1 cells with a higher frequency of $CD4^-CD8^+$ cells, while in the last stage, stage 3, cells consist of almost only $CD4^-$ $CD8^+$ cells indicating that MAIT cells develop from DP cells to CD8SP cells in humans. In the last stage of thymic development, the expression of common MAIT cell surface proteins like IL-18R and CD161 was upregulated, indicating that stage 3 MAIT cells have a appearance that is similar to MAIT cells that can be found in human blood (Koay *et al.*, 2016). Besides IL-18R and CD161, also PLZF is upregulated in stage 3 MAIT cells. Analysis of PLZF-null mice showed that MAIT cells could not develop into stage 3 MAIT cells when PLZF is missing in these mice, indicating that PLZF is a factor that regulates transition from stage 2 to stage 3 MAIT cells.

MAIT cells that are found within the thymus are mostly stage 1 and 2 MAIT cells, while only a small population of stage 3 cells is present in the thymus. In contrast, in the periphery, where MAIT cells undergo further maturation and expansion, mainly stage 3 MAIT cells are present but only a small number of stage 2 MAIT cells.

During investigation of MR1 tetramer reactivity in human thymus samples, a population of MR1 tetramer$^+$ cells was identified that is CD4$^+$ CD8$^+$ double positive. Furthermore, these cells had a distinct transcription factor expression profile compared to MAIT cells. They did not express PLZF but the regulatory T cell associated transcription factor FOXP3. A still open question in the field of MAIT cell research is how MAIT cells were regulated, especially in the intestine, since our microflora contains many MAIT cell-activating ligands. Regulatory T (T_{reg}) cells are normally important in regulating immune responses and express FOXP3. Interestingly, the cells identified here also express FOXP3, a transcription factor that is linked to T_{regs}. This made these here identified MR1 tetramer$^+$ FOXP3$^+$ cells of high interest, because they could be a regulatory subset of MR1-reactive T cells. The aim of this chapter was therefore to characterize these MR1 tetramer$^+$ cells in human thymus for their transcription factor expression, as well as surface protein expression and their TCR usage.

5.2. Results

5.2.1. Identification of FOXP3$^+$ T-bet$^+$ MR1-reactive T cells

The investigation of MR1-tetramer reactivity within the human thymus revealed a population of MR1-reactive T cells that lacked Vα7.2, the variable TCRα chain segment most commonly used by MAIT cells (Tilloy *et al.*, 1999), as well as PLZF expression (Fig. 5.1). The lack of PLZF expression, might indicate that the cells were immature MAIT cells that have not gained PLZF expression yet (Koay *et al.*, 2016), but the lack of Vα7.2 expression indicated that they are not classical MAIT cells. Besides PLZF and Vα7.2 expression, it was shown that MR1-5-OP-RU tetramer$^+$ cells were negative for RORγT. In contrast, developing DP thymocytes are usually RORγT$^+$ (Koay *et al.*, 2016), since RoRγT is necessary for the survival and development of DP thymocytes (Sun *et al.*, 2000), implying that MR1-5-OP-RU tetramer$^+$ cells were not developing cells. Furthermore, MAIT cell populations of atypical MR1-restricted T cells were described as MR1

tetramer$^+$ cells that were PLZF$^-$ and Vα7.2$^-$ (Gherardin *et al.*, 2016; Lepore *et al.*, 2017; Harriff *et al.*, 2018). Whilst atypical MR1-resticted T cells are rare (Gherardin *et al.*, 2016; Lepore *et al.*, 2017; Harriff *et al.*, 2018), the aforementioned subset was relatively abundant (Fig. 5.1). During further analysis of MR1 tetramer$^+$ Vα7.2$^-$ T cells and their transcription factors, they were identified as CD4$^+$ T cells that co-expressed the transcription factors forkhead box P3 (FOXP3) as well as T box transcription factor (T-bet) (Fig. 5.2 A). T-bet is known to be a regulator of T_H1 cells (Szabo *et al.*, 2000), and is expressed by MAIT cells as well (Koay *et al.*, 2016; Gherardin *et al.*, 2018), whereas FOXP3 is known to be a master regulator of T_{regs} (Fontenot *et al.*, 2005). So far no expression of FOXP3 by MR1 tetramer$^+$ cells have been reported. However, the co-expression of FOXP3 and T-bet has been reported in Th1-like T_{regs} following peripheral activation (Duhen *et al.*, 2012), and amongst T_{regs} that recirculated to the human thymus (Thiault *et al.*, 2015).

Approximately 2.00 % of CD4$^+$ T cells co-expressed the transcription factors FOXP3 and T-bet (Fig. 5.2 A,B). In contrast, MAIT cells are less frequent in human thymus (< 0.05 %) (Koay *et al.*, 2016). FOXP3$^+$ T-bet$^+$ T cells clearly bound MR1 5-OP-RU tetramer, whilst displaying a negative bias for Vα7.2 expression in comparison to the bulk T cell pool (Fig. 5.3), suggesting that the FOXP3$^+$ T-bet$^+$ cells have a different TCR usage than MAIT cells. Comparison of CD4$^+$ T cell subsets based on their FOXP3 and T-bet expression revealed that MR1-reactivity was heavily biased towards FOXP3$^+$ T-bet$^+$ population (Fig.5.2 C). Furthermore, a linear correlation between CD8α expression and MR1 tetramer staining was observed, suggesting a potential role for CD8α in the binding of MR1 tetramers (Fig. 5.2 C). Autofluorescent and dead cells were excluded by blotting a viability dye against an empty flow cytometry channel. As autofluorescence channel, the filter 525/20 of the violet laser (405 nm) was used. All dead and autofluorescent cells were excluded and cells were further gated on CD3. The complete gating strategy is shown in appendix figure 2. Additionally, PBMCs were analyzed for correlation of MR1-5-OP-RU tetramer staining

against CD8α. Representative FACS plots are shown in appendix figure 3. Here staining of MR1-5-OP-RU tetramer$^+$ CD8α$^+$ cells show a tendency of a diagonal staining, indicating that also here CD8α can play a possible role in MR1 tetramer binding.

Using MR1-6FP tetramer in human thymus, a similar staining pattern as for MR1-5-OP-RU tetramer was observed, but a lower frequency of MR1-6FP tetramer$^+$ cells was noted compared to MR1-5-OP-RU tetramer$^+$ cells (Fig. 5.2 D). Around 17.85 % FOXP3$^+$ T-bet$^+$ T cells bound to the MR1-5-OP-RU tetramer, whereas only 9.1 % bound to the MR1-6-FP tetramer (Fig. 5.2 E). This stands in contrast to normal MAIT cells that bound to MR1-5-OP-RU tetramer, but failed to recognize MR1 loaded with 6-FP (Fig. 5.4). Using MR1-5-OP-RU tetramer, a positive population was identified within CD3$^+$ cells that co-expressed Vα7.2 and represented MAIT cells. In contrast, MR1-6-FP tetramers did not stain MAIT cells and no cell population was observed using MR1-6-FP tetramer. This stands in contrast to the FOXP3$^+$T-bet$^+$ CD4$^+$ T cells found within the human thymus, where both tetramers could bin to the cells (Fig. 5.4). Comparing the two different tetramers, the frequency of MR1-6-FP tetramer$^+$ cells was lower than the frequency of MR1-5-OP-RU tetramer$^+$ cells. This indicated that these FOXP3$^+$ T-bet$^+$ T cells could recognize MR1 in the context of both ligands, in contrast to MAIT cells that only recognize MR1 tetramers loaded with 5-OP-RU. Whether these differences are indicative of differential antigen affinity has yet to be resolved, but these data do suggest that the antigen-specificity of FOXP3$^+$ T-bet$^+$ cells may differ from that of classical MAIT cells.

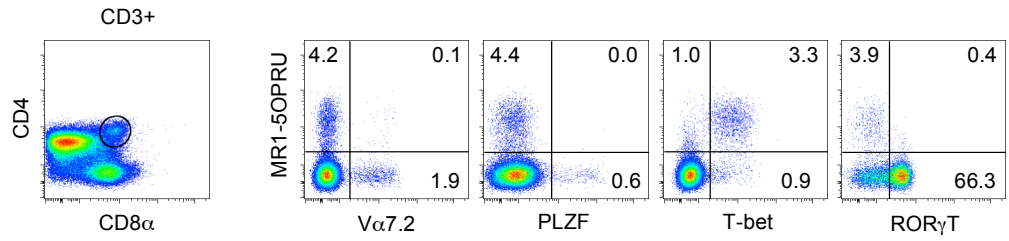


Figure 5. 1 – Identification of MR1-5-OP-RU tetramer$^+$ cells in human thymus. Representative FACS plots of CD3$^+$ cells gated on CD4$^+$ and CD8$^+$ cells. DP (CD4$^+$ CD8$^+$) cells were further analyzed for MR1-5-OP-RU tetramer staining against Vα7.2, PLZF, T-bet and RORγT.

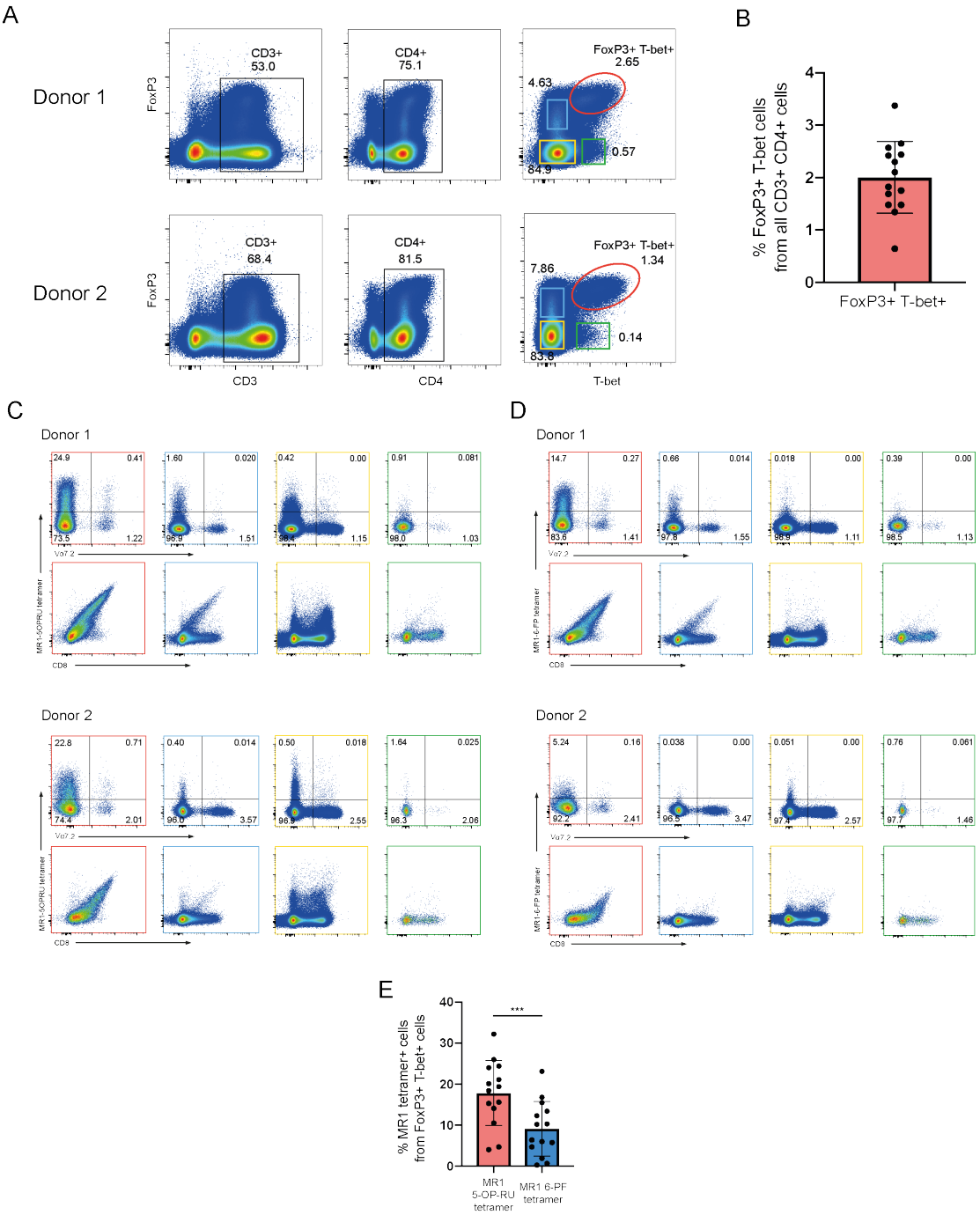


Figure 5. 2 – FOXP3^{+} T-bet^{+} T cells in human thymus. (A) Gating of FOXP3+ T-bet+ T cells in human thymus represented by two different donors. (B) Percenatge of FOXP3^{+} T-bet^{+} T cells of all analyzed donors (n=14). (C) Gating of MR1-5-OP-RU tetramer against Vα7.2 and CD8α for the different populations FOXP3^{+} T-bet^{+}, FOXP3^{+}, FOXP3^{-} T-bet^{-} and T-bet^{+} for the two representative donors of A. (D) Gating of MR1-6-FP tetramer against Vα7.2 and CD8α for the different populations FOXP3^{+} T-bet^{+}, FOXP3^{+}, FOXP3^{-} T-bet^{-} and T-bet^{+} for the two representative donors of A. (E) Percentage of MR1-5-OP-RU tetramer and MR1-6-FP tetramer positive T cells from FOXP3^{+} T-bet^{+} T cells (n=14). ***P≤0.001 using Wilcoxon matched-pairs signed rank test.

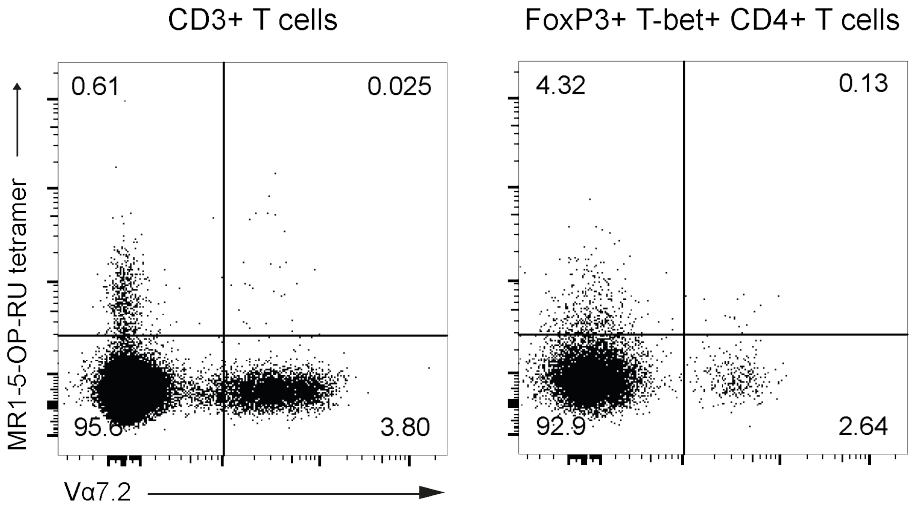


Figure 5. 3 – FOXP3$^+$ T-bet$^+$ CD4$^+$ T cells display a negative bias for Vα7.2. Representative FACS plots of CD3$^+$ or FOXP3$^+$ T-bet$^+$ CD4$^+$ T cells from one human thymus showed MR1-5-OP-RU tetramer staining against Vα7.2.

To isolate viable FOXP3$^+$ T-bet$^+$ cells for functional studies or for TCR sequencing analyses, surface-receptors that could act as surrogate markers for FOXP3 and T-bet were examined. Expression of CD4 and CD25, also known as IL-2 receptor alpha chain, is commonly used to identify FOXP3+ T_{regs} (Sakaguchi *et al.*, 1995; Fontenot *et al.*, 2005; Wu *et al.*, 2006). Expression of CD127, also known as IL-7Rα, has been associated with T-bet$^+$ cells, but some studies reported that CD127 expression inversely correlates to expression of T-bet (Colpitts *et al.*, 2009; Carrette and Surh, 2012; Knox *et al.*, 2014). Due to this, CD25 and CD127 co-expression was investigated using the same gating strategy as for FOXP3$^+$ T-bet$^+$ cells. As shown in figure 5.5 A, a clear population of CD4$^+$ T cells co-expressing CD25 and CD127 was identified within human thymus. This population showed a frequency of 1.9 % CD25$^+$ CD127$^+$ cells from all CD4$^+$ T cells (Fig 5.5. B). This finding is comparable to the FOXP3$^+$ T-bet$^+$ cells, which showed a similar frequency within all CD4$^+$ T cells (Fig 5.2 B). The CD25$^+$ CD127$^+$ CD4$^+$ T cells could bind MR1 tetramer (Fig 5.5 C) in a similar manner to the FOXP3$^+$ T-bet$^+$ cells (Fig 5.2 C). Comparing the CD25$^+$ CD127$^+$ subset to the single positive populations (CD25$^+$ or CD127$^+$) as well

as to the $CD25^-$ $CD127^-$ cells, it was shown that the highest percentage of MR1-reactive cells was found within the $CD25^+$ $CD127^+$ population (Fig. 5.5 C). Here, the same correlation of MR1 tetramer and CD8α expression was observed as for $FOXP3^+$ $T\text{-}bet^+$ T cells (Fig. 5.1 C). Besides MR1-5-OP-RU tetramer, also the MR1-6-FP tetramer could bind to $CD25^+$ $CD127^+$ cells in correlation to CD8α expression (Fig. 5.5 D). 20.57 % of all $CD25^+$ $CD127^+$ T cells were MR1-5-OP-RU tetramer positive. In contrast only 10.52 % were MR1-6FP tetramer positive (Fig. 5.5 E). Like $FOXP3^+$ $T\text{-}bet^+$ cells, $CD25^+$ $CD127^+$ showed a higher frequency of MR1-5-OP-RU $tetramer^+$ cells than MR1-6FP $tetramer^+$ cells. The similar binding of MR1 tetramers suggested that $CD25^+CD127^+$ were similar or the same cells as $FOXP3^+T\text{-}bet^+$ cells. To confirm that CD25 and CD127 were reliable markers for identification of

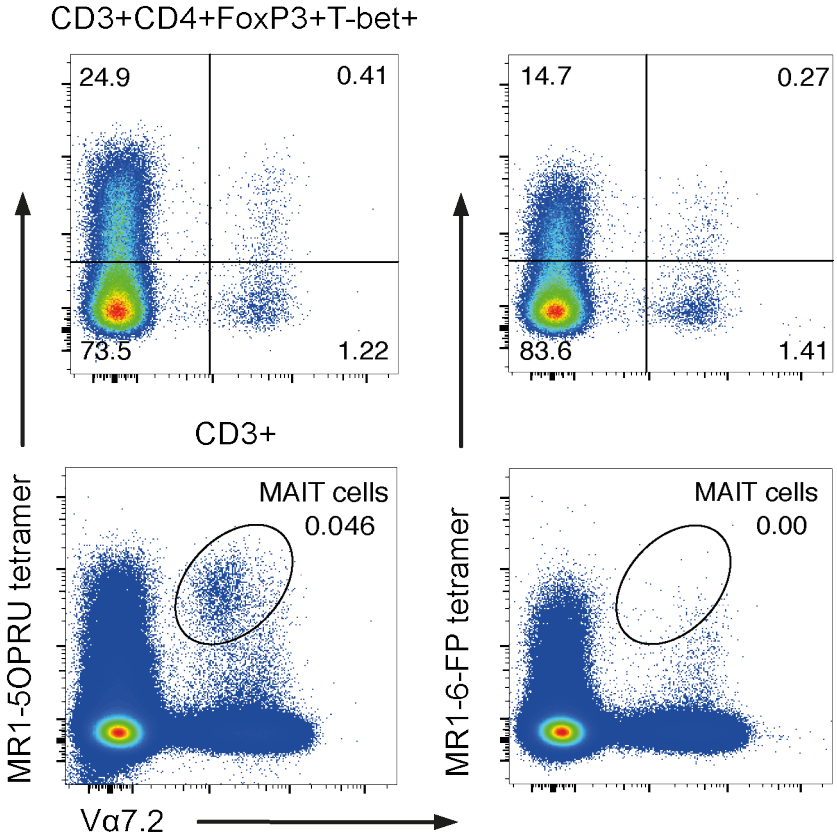


Figure 5. 4 – Comparison of MR1 tetramer staining on $FOXP3^+$ $T\text{-}bet^+$ $CD4^+$ T cells and $CD3^+$ T cells in human thymus. Representative plots of MR1 tetramer staining. The upper plots show MR1-5-OP-RU or MR1-6-FP tetramer staining against Vα7.2 on $CD3^+CD4^+FOXP3^+T\text{-}bet^+$ cells, while the lower blots show MR1-5-OP-RU or MR1-6-FP tetramer staining against Vα7.2 on $CD3^+$ cells in human thymus

FOXP3$^+$ T-bet$^+$ cells, a co-staining of the transcription factors FOXP3 and T-bet as well as the corresponding surface markers CD25 and CD127 was performed. Within the population of FoxP3$^+$ T-bet$^+$ T cells, 74.1 % CD25$^+$ CD127$^+$ cells could be found. During analysis of CD25$^+$CD127$^+$ T cells, 75.4 % of the CD25$^+$CD127$^+$ T cells co-expressed FoxP3 as well as T-bet. Gating on CD25$^+$ CD127$^+$ cells showed a clear population of FoxP3$^+$ T-bet$^-$ T cells. This indicated that whilst assessing CD25 and CD127 co-expression may enrich for FOXP3$^+$ T-bet$^+$ cells, it might not be definitive (Fig. 5.6). This could be due to the fact that the different proteins were not exclusively expressed on the same cells. Another explanation for the T-bet$^-$ population within CD25$^+$ CD127$^+$ cells could be due to an inferior antibody staining of CD25 and CD127. The CD127 antibody used in this experiment was conjugated to the fluorochrome PerCP-Cy5.5, which has a moderate brightness. This could explain the weak CD127 staining in this experiment. Furthermore, a lower affinity of the CD127-PerCP-Cy5.5 antibody could explain the differences in staining compared to the antibody used in figure 5.5, which is a different antibody clone conjugated with PE-Cy7. Since most of CD25$^+$ CD127$^+$ cells co-expressed FOXP3 and T-bet, CD25 and CD127 were used as alternative markers for FoxP3 and T-bet to perform functional assays and to determine the TCR usage.

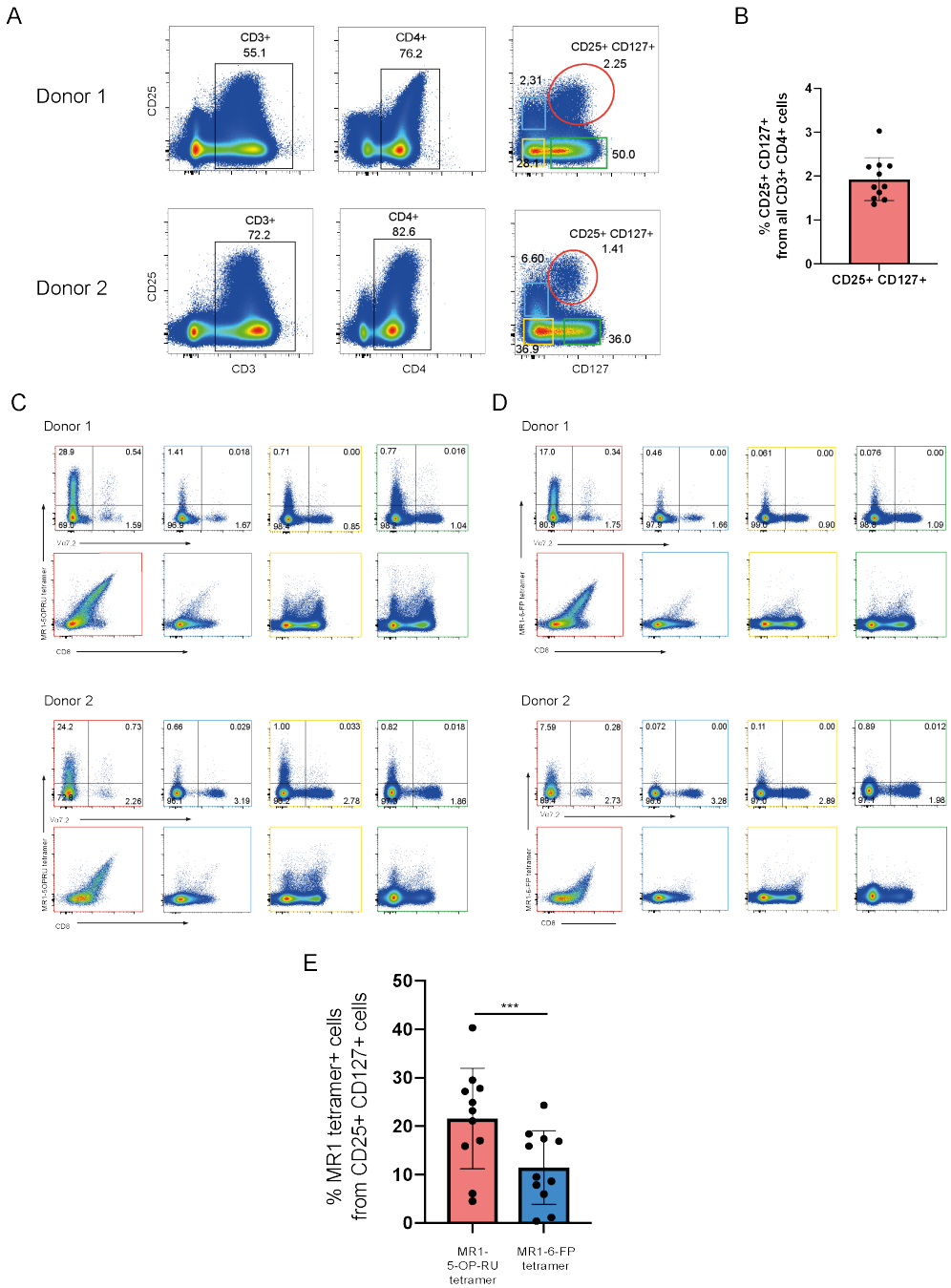


Figure 5.5 – CD25$^+$ CD127$^+$ T cells in human thymus. (A) Gating of CD25$^+$ CD127$^+$ T cells in human thymus represented by two different donors. The plots CD25 against CD127 show less events for better visibility of populations. (B) Percenatge of CD25$^+$ CD127$^+$ T cells of all analyzed donors (n=11). (C) Gating of MR1-5-OP-RU tetramer against Vα7.2 and CD8α for the different populations CD25$^+$ CD127$^+$, CD25$^+$, CD25$^-$ CD127$^-$ and CD127$^+$ for the two representative donors of A. (D) Gating of MR1-6-FP tetramer against Vα7.2 and CD8α for the different populations CD25$^+$ CD127$^+$, CD25$^+$, CD25$^-$ CD127$^-$ and CD127$^+$ for the two representative donors of A. (E) Percentage of MR1-5-OP-RU tetramer and MR1-6-FP tetramer positive T cells from CD25$^+$ CD127$^+$ T cells (n=11). ***P≤0.001 using Wilcoxon matched-pairs signed rank test.

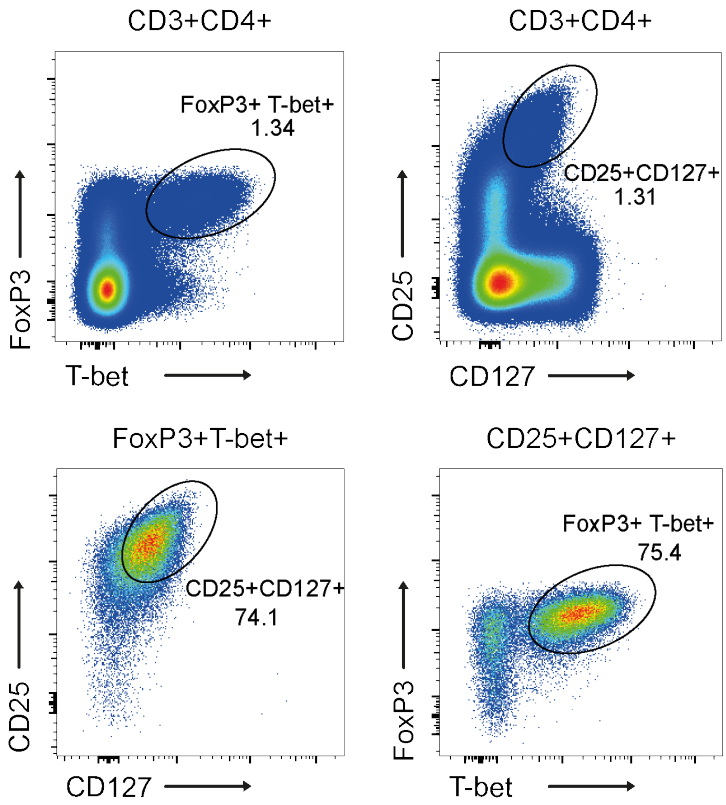


Figure 5. 6 – Co-staining of FOXP3, T-bet as well as CD25 and CD127 on human thymus samples. Human thymocytes were gated on live $CD3^+$ $CD4^+$ T cells. Cells were further gated for either $FOXP3^+$ $T\text{-}bet^+$ cells or $CD25^+$ $CD127^+$ cells. $FOXP3^+$ $T\text{-}bet^+$ T cells were analyzed for CD25 and CD127 expression, while $CD25^+$ $CD127^+$ T cells were analyzed for FOXP3 and T-bet expression. The data is representative of one donor.

The thymuses used in these experiments originated from children with an age range from 6 days to 15 years. Interestingly, the frequency of $FOXP3^+$ $T\text{-}bet^+$, or $CD25^+$, $CD127^+$ cells that were MR1-tetramer positive correlated with donor age. For example, a positive correlation (r=0.4022, P=0.16) between $FoxP3^+$ $T\text{-}bet^+$ MR1-5-OP-RU $tetramer^+$ and donor age was identified (Fig 5.7 B). A similar positive correlation was found between $CD25^+$ $CD127^+$ MR1-5-OP-RU $tetramer^+$ cells and the age of the donors (r=0.4, P=0.22) (Fig. 5.7 D). Even though the correlations were positive no

significance was noted. No correlation between frequency of FOXP3$^+$T-bet$^+$ T cells and age was found. The same was observed for frequency of CD25$^+$CD127$^+$ T cells (Fig 5.7 A, C).

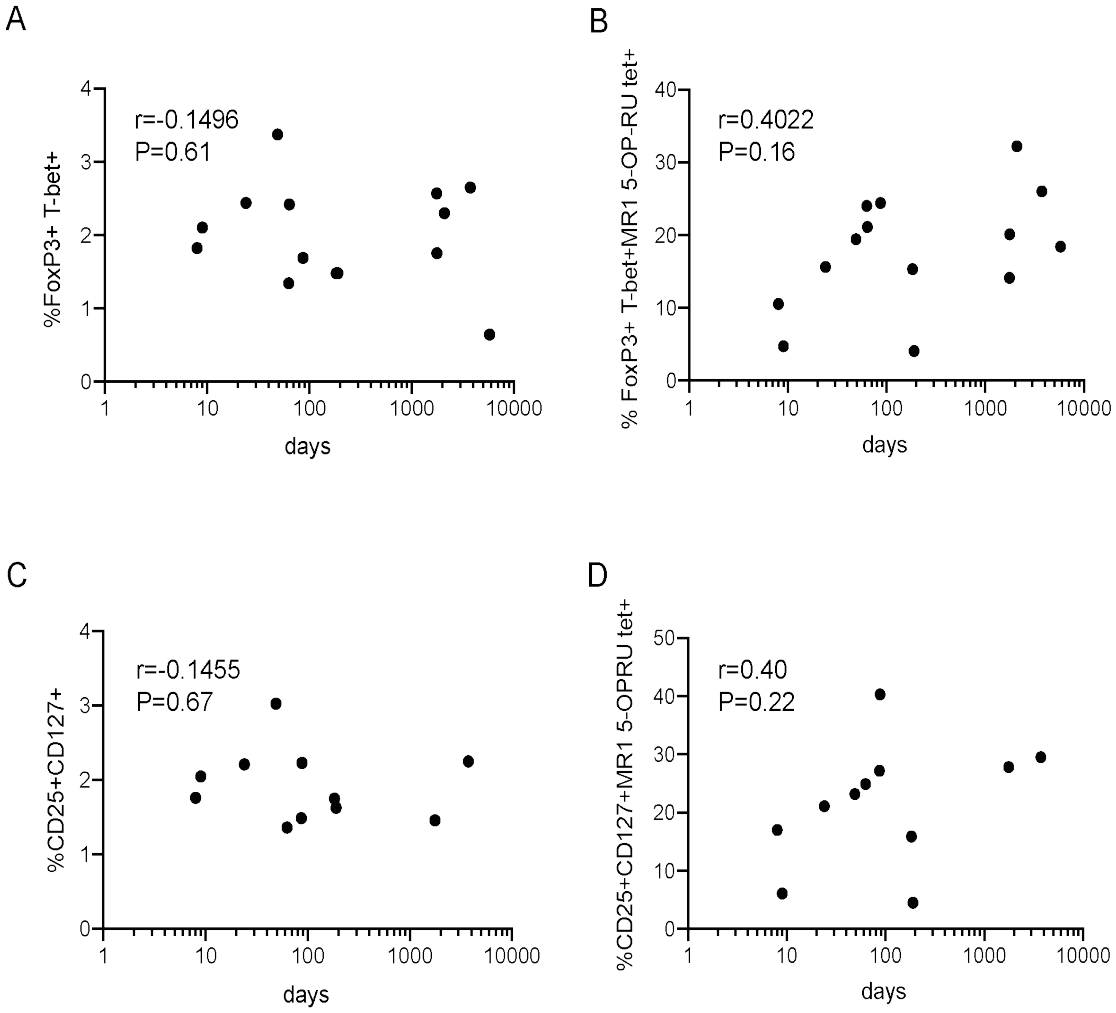


Figure 5. 7 – Correlation of FOXP3$^+$ T-bet$^+$ or CD25$^+$ CD127$^+$ T cells and donor age. (A) Correlation between percentage of FOXP3$^+$ T-bet$^+$ T cells and age of donors. (B) Correlation between percentage of FOXP3$^+$ T-bet$^+$ MR1-5-OP-RU tetramer$^+$ cells and age of donors. (C) Correlation between percentage of CD25$^+$CD127$^+$ of all CD4$^+$ T cells and age of donors. (D) Correlation between percentage of CD25$^+$ CD127$^+$ MR1-5-OP-RU tetramer$^+$ cells and age of donors. All correlations were analyzed using spearman correlation.

5.2.2. Phenotypic analysis of FOXP3+ T-bet+ T cells and CD25+ CD127+ T cells

For characterization of FOXP3$^+$ T-bet$^+$ T cells, the expression of different immune-related proteins was analyzed. The proteins cytotoxic T lymphocyte associated antigen 4 (CTLA-4), glucocorticoid induced tumor necrosis factor (TNF) receptor related protein (GITR) as well as the inducible co-stimulatory molecule (ICOS) were assessed as these are known to be expressed by FOXP3$^+$ T_{regs} (Ito *et al.*, 2008; Rodríguez-Perea *et al.*, 2016). CTLA-4 as well as GITR are also expressed during the development of thymic T_{regs} (Annunziato *et al.*, 2002; Cosmi *et al.*, 2003; Cupedo *et al.*, 2005), but no reports of MAIT cells expressing CTLA-4 and GITR has been reported. For the T_{reg} associated protein ICOS, two distinct populations were found within human thymus, ICOS$^-$ and ICOS$^+$ T cells (Ito *et al.*, 2008). CD103, also known as integrin alpha E, was analyzed as well. It is expressed on DP thymocytes and CD8SP cells during T_{reg} development (Nunes-Cabaço *et al.*, 2011). During analysis of murine thymus samples, CD103 as well as ICOS expression was low on immature stage 1 MAIT cells, while stage 3 MAIT cells showed expression of ICOS and CD103 (Koay *et al.*, 2016).

The regulatory T cell markers ICOS, GITR and CTLA-4 were expressed on FOXP3$^+$T-bet$^+$ cells, while CD103 was not expressed on these cells. Analyzing FOXP3$^+$ T cells in human thymus, only CTLA-4 showed clear expression, while ICOS and GITR were not detected in the FOXP3$^+$ population, which stands in contrast to reports about T_{reg} development (Annunziato *et al.*, 2002; Cosmi *et al.*, 2003; Cupedo *et al.*, 2005; Nunes-Cabaço *et al.*, 2011). Looking at CD103 expression, only a very small population of CD103$^+$ cells was found within FOXP3$^+$ cells (Fig. 5.8), but expression on CD8$^+$ T cells was observed.

Besides the common regulatory T cell markers described above, the FOXP3$^+$ T-bet$^+$ T cell subset was also assessed for its chemokine receptor expression. The chemokine receptors CCR5, CCR6 and CCR7 were analyzed here. CCR5 is expressed on lymphocytes in various

tissues (Kunkel *et al.*, 2002), whereas CCR6 is associated with homing of T cells in mucosal tissues like the airways as well as homing of T_{regs} in the gut and kidney (Thomas *et al.*, 2007; Cook *et al.*, 2014; Krebs *et al.*, 2016). CCR7 is known to be important for homing of T cells to secondary lymphoid organs (Campbell and Butcher, 2000), but it is also expressed on SP thymocytes as well as some DP thymocytes and play a role in migration within the thymus (Witt and Robey, 2004). These chemokine receptors were used to get an idea where $FOXP3^+$ $T\text{-}bet^+$ could be located after development. $FOXP3^+$ $T\text{-}bet^+$ T cells expressed CCR5 and CCR6 but not CCR7. Only $T\text{-}bet^+$ cells expressed CCR5 and CCR6 while some staining of CCR7 was observed in $T\text{-}bet^+$, $FOXP3^-$ $T\text{-}bet^-$ and $FOXP3^+$ cells but not $FOXP3^+$ $T\text{-}bet^+$ cells (Fig.5.8).

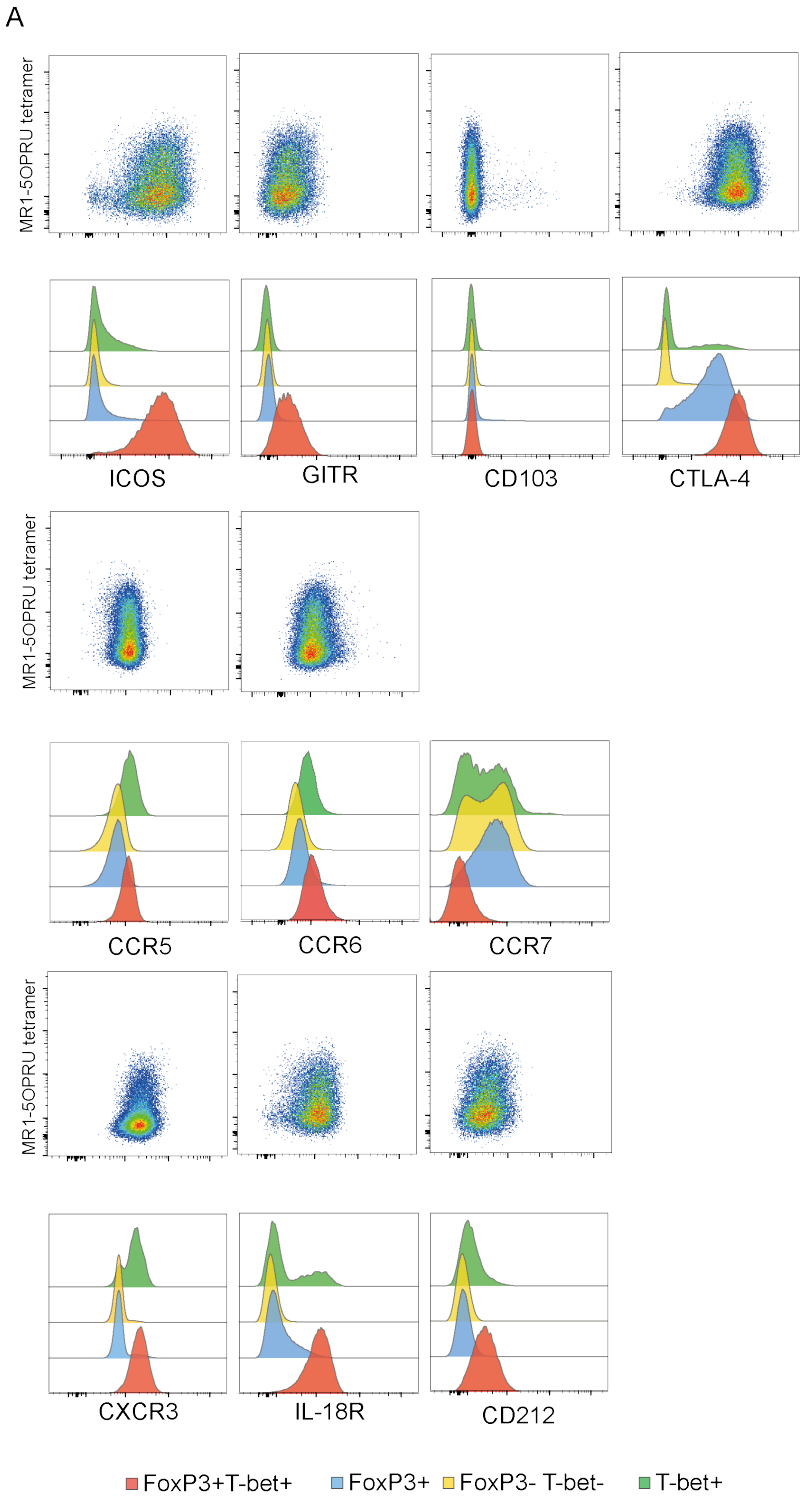


Figure 5. 8 - Phenotyping of FOXP3^{+} T-bet^{+} T cells. (A) CD4^{+} thymocytes were divided into subsets based on gating in figure 5.2. The populations FOXP3^{+}T-bet^{+}, FOXP3^{+}, FOXP3^{-} T-bet^{-} and T-bet^{+} were analyzed for the different markers ICOS, GITR, CD103, CTLA-4, CCR5, CCR6, CCR7, CXCR3, IL-18R and CD212. The shown histograms are representatives of 2-3 experiments. FACS plots showing staining of MR1-5OP-RU tetramer versus the indicated markers on FoxP3^{+} T-bet^{+} T cells.

Further analysis showed expression of CXCR3, IL-18R and CD212 on FOXP3$^+$T-bet$^+$ T cells. CXCR3 is known to be expressed on T_H1 CD4$^+$ T cells and effector CD8$^+$ T cells and enables the migration into inflamed peripheral tissues (Groom and Luster, 2011). IL-18R is expressed on T_H1 cells as well as MAIT cells (Yoshimoto *et al.*, 1998; Koay *et al.*, 2016), while CD212 (IL-12R) is expressed on T_H1 cells as well (Rogge *et al.*, 1997). CD212 is also expected to be expressed by MAIT cells, since they respond to IL-12 (Ussher *et al.*, 2014). Here, T-bet$^+$ cells also showed expression of CXCR3 and IL-18R. All in all, this indicated that FOXP3$^+$ T-bet$^+$ T cells were a distinct population to FOXP3$^+$ or T-bet$^+$ T cells or FOXP3$^-$ T-bet$^-$ T cells. Furthermore, it indicated that FOXP3$^+$ T-bet$^+$ T cells expressed proteins associated with MAIT cells, but seem to be a distinct population to MAIT cells. Moreover, analysis of FOXP3+ T-bet+ subset revealed that the MR1-tetramer$^+$ fraction only differed in terms of their CD8α expression since MR1-tetramer$^+$ cells expressed the markers analyzed here in a similar fashion, as did MR1-tetramer$^-$ cells (Fig. 5.8). This indicated that the MR1 tetramer$^+$ cells were not a distinct population within the FOXP3$^+$T-bet$^+$ cells. The only difference between MR1-5-OP-RU tetramer positive and negative cells so far observed was their CD8α expression (Fig. 5.2 C).

The phenotype of CD25$^+$ CD127$^+$ T cell subset was analyzed as well. As shown in figure 5.9, CD25$^+$ CD127$^+$ T cells expressed ICOS, GITR as well as CTLA-4, but not CD103, similar to FOXP3$^+$ T-bet$^+$ cells shown before (Fig. 5.8). CD25$^+$ CD127$^+$ also expressed CCR6 but no CCR7 similar to FOXP3$^+$ T-bet$^+$ cells (Fig.5.8). Additionally, CCR4 and CCR9 were analyzed. CCR4 is known to be expressed in T_H2 cells and T_{regs}, while CCR9 is expressed on thymocytes as well as IELs and is important for gut-homing (Imai *et al.*, 1999; Zabel *et al.*, 1999; Iellem *et al.*, 2001). Studies in mice showed that early DN as well as DP expressed CCR9 and that CCR9 was downregulated on SP thymocytes (Uehara *et al.*, 2006). Here, CD25$^+$ CD127$^+$ T cells showed expression of CCR4 but not CCR9, suggesting that CD25$^+$ CD127$^+$ T cells were not recruited to the gut after development, but it

cannot be excluded that cells will express this chemokine receptor later during development.

Analysis of CXCR3 and IL-18R, showed high expression of CXCR3 and IL-18R similar to what was previously shown for FOXP3$^+$ T-bet$^+$ T cells. Additionally, CD45RA and CD45RO were analyzed. It is known, that CD45RO is expressed by positively selected thymocytes, while it is downregulated before emigration from the thymus simultaneously to CD45RA upregulation. This indicated that CD45RO$^+$ T cells show a more immature phenotype than the cells that already lost CD45RO expression (Fujii *et al.*, 1992; Fukuhara *et al.*, 2002). In the periphery, CD45RA is expressed on naïve T cells, while CD45RO is expressed upon antigen stimulation and on memory T cells (Michie *et al.*, 1992). CD25$^+$ CD127$^+$ T cells showed expression of CD45RO, but not CD45RA, indicating that they are immature T cells in the thymus.

In addition, the phenotype of MR1 tetramer$^+$ cells was similar to the whole CD25$^+$ CD127$^+$ population as shown in figure 5.9, which indicated that also here, MR1 tetramer$^+$ cells were not a distinct population within CD25$^+$ CD127$^+$ cells, similar to FOXP3$^+$ T-bet$^+$ cells.

This indicated that both the FOXP3$^+$ T-bet$^+$ and CD25$^+$ CD127$^+$ cell subsets share multiple phenotypic similarities and are distinguishable from other T cell subsets within the thymus.

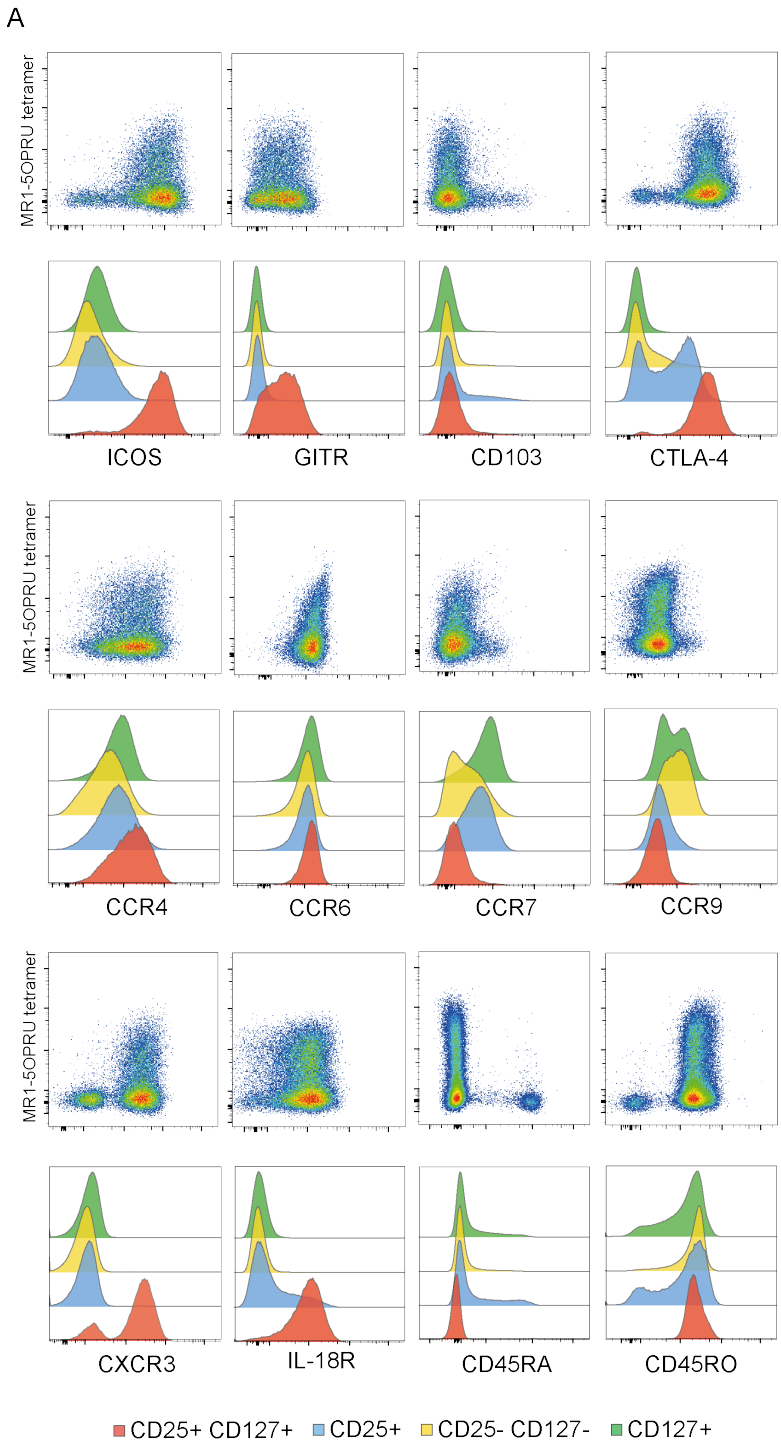


Figure 5. 9 – Phenotyping of CD25^{+} CD127^{+} T cells. (A) CD4^{+} thymocytes were divided in subsets based on gating in figure 5.5. The populations CD25^{+}CD127^{+}, CD25^{+}, CD25^{-} CD127^{-} and CD127^{+} were analyzed for the different markers ICOS, GITR, CD103, CTLA-4, CCR4, CCR6, CCR7, CCR9, CXCR3, IL-18R, CD45RA and CD45RO. The shown histograms are representatives of 2-3 experiments. FACS plots showing staining of MR1-5OP-RU tetramer versus the indicated markers on CD25^{+} CD127^{+} T cells.

Comparison of $CD25^+$ $CD127^+$ T cell subset to MAIT cells, revealed differences in their phenotype to classical MAIT cells. For example, thymic MAIT cells lacked expression of ICOS, GITR or CTLA-4, while some MAIT cells expressed CD103 (Fig. 5.10). Murine data showed, that MAIT cells express low levels of CD103 within the thymus and express ICOS in an immature state, while they express ICOS and CD103 at stage 3 of development (Koay *et al.*, 2016). At the time, no human data is available on ICOS, CTLA-4, CD103 and GITR expression by MAIT cells in thymus samples. Whilst analyzing chemokine receptor expression, MAIT cells maintained lower levels of CCR4, yet had similar levels of CCR5, CCR6 and CCR9 compared to the $CD25^+$ $CD127^+$ subset (Fig. 5.10). Previous reports reported that MAIT cells in peripheral blood mononuclear cells (PBMCs) are negative for CCR4 and CCR9 and positive for CCR5 and CCR6 (Dusseaux *et al.*, 2011; Gherardin *et al.*, 2018), but in thymuses, only around 10 % of MAIT cells showed a mature phenotype that was phenotypically similar to MAIT cells found in PBMCs. In contrast, MAIT cells showed CCR7 expression while $CD25^+$ $CD127^+$ $CD4^+$ T cells were negative for CCR7. In PBMCs, it was shown that MAIT cells were negative for CCR7 (Dusseaux *et al.*, 2011; Gherardin *et al.*, 2018), but this may not represent the phenotype of MAIT cells in the thymus. Only a few MAIT cells showed CXCR3 expression, while most $CD25^+$ $CD127^+$ CD4 T cells expressed CXCR3 (Fig. 5.10). Additionally, IL-18R expression analysis showed, that only few MAIT cells that express IL-18R. These IL-$18R^+$ MAIT cells could represent stage 3 MAIT cells, while stage 1 and 2 MAIT cells expressed no IL-18R, as reported by Koay et al. (Koay *et al.*, 2016). In contrast, $CD25^+$ $CD127^+$ $CD4^+$ T cells were positive for IL-18R (Fig. 5.10). The expression of CD45RA and CD45RO was similar between MAIT cells and $CD25^+$ $CD127^+$ T cells (Fig 5.10). One study in human thymus observed that half of the MAIT cells expressed CD45RA (Martin *et al.*, 2009). This indicated that MAIT cells in thymus are mostly immature, which was also shown by Koay et al. Taken these findings together, it seems like MAIT cells and $CD25^+$

CD127$^+$ T cells were distinct cell populations within the thymus, but they share expression of particular markers.

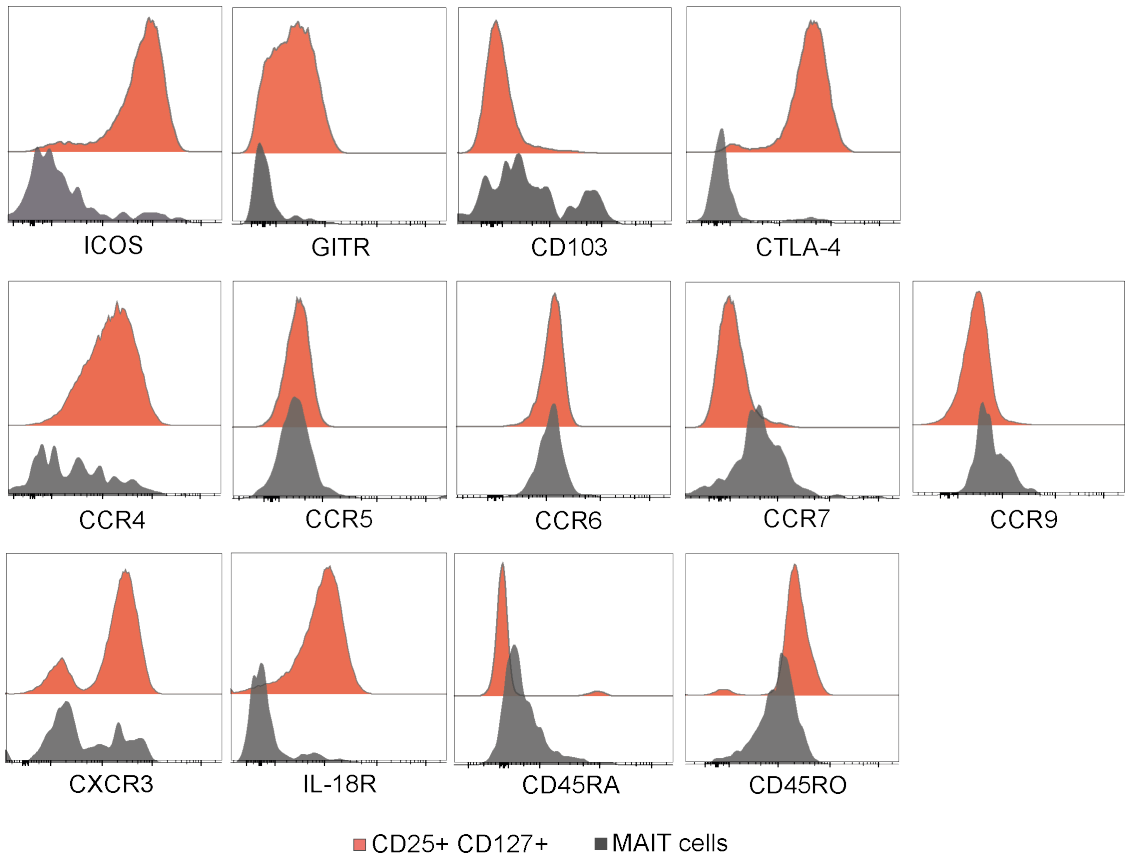


Figure 5. 10 – Expression of previously analyzed markers on CD25$^+$CD127$^+$ T cells and MAIT cells. Representative histograms of ICOS, GITR, CD103 and CTLA-4 on CD25$^+$CD127$^+$ CD4$^+$ T cells and MAIT cells in human thymus are shown. Furthermore, histograms of CCR4, CCR5, CCR6, CCR7 and CCR9 on CD25$^+$CD127$^+$ CD4$^+$ T cells and MAIT cells in human thymus, as well as histograms of CXCR3, IL-18R, CD45RA and CD45RO on CD25$^+$CD127$^+$ CD4$^+$ T cells and MAIT cells. MAIT cells were gated as MR1-5-OP-RU tetramer$^+$ Vα7.2$^+$ cells within CD3$^+$ T cells.

5.2.3. TCR usage of CD25$^+$CD127$^+$ CD4$^+$ T cells

To investigate the TCR usages of the CD25$^+$ CD127$^+$ T cells, single cells were sorted *ex vivo* by flow cytometry from human thymus samples. Cells were initially isolated based on their positive expression of CD3, CD4,

CD25 and CD127, with those being positive or negative for MR1-5-OP-RU tetramer being segregated. After sorting, the TCR was sequenced by multiplex PCR. Therefore, cDNA was synthesized of the sorted single cells and two rounds of nested PCRs were performed to reduce non-specific binding. Two sets of primer were used therefore with the first primer pairs upstream of the second primer set. The resulting amplicons from the first PCR were used as templates for the second PCR run with the second primer pairs. Here, only primers could bind to the wanted target sequence, while the primers should not bind to unwanted products of the first PCR round, leading to reduction of non-specific sequences. After the nested PCRs, primers and nucleotides of excess were hydrolyzed. The clean amplified PCR product was sequenced afterwards. Analyzing the αβ gene usage of MR1-5-OP-RU tetramer$^+$ cells, a high diversity of TRAV genes was found within and between donors, but with some TRAV genes were used more often e.g. TRAV5 in donor 2 and 3. Comparing MR1-5-OP-RU tetramer$^+$ to MR1-5-OP-RU tetramer$^-$ cells a similar diversity of TRAV genes was observed, but also here some genes were used more often than others e.g. TRAV6, TRAV17 and TRAV20. TRAV genes that were used more frequent differed between MR1-5-OP-RU tetramer$^+$ and MR1-5-OP-RU tetramer$^-$ cells (Fig.5.11).

The TRAJ gene usage of CD25$^+$ CD127$^+$ cells showed a diverse repertoire between and within donors. Here, no differences between MR1-5-OP-RU positive or negative cells were observed. This indicated that the α-chain of CD25$^+$ CD127$^+$ MR1-5-OP-RU$^+$ cells is very diverse and not invariant as the α-chain of MAIT cells (Fig5.12).

Analyzing the TRBV gene usage, a diverse repertoire was identified despite some biases for MR1-5-OP-RU tetramer$^+$ cells (TRBV28 and TRBV12-4) and MR1-5-OP-RU tetramer$^-$ cells (TRBV20-1) (Fig. 5.13) being observed. Altogether, this highlights the diverse αβ-chain usages amongst the CD25$^+$CD127$^+$ T cell subset, which stands in contrast to the restricted TCR gene usages reported for MAIT cells (Tilloy *et al.*, 1999).

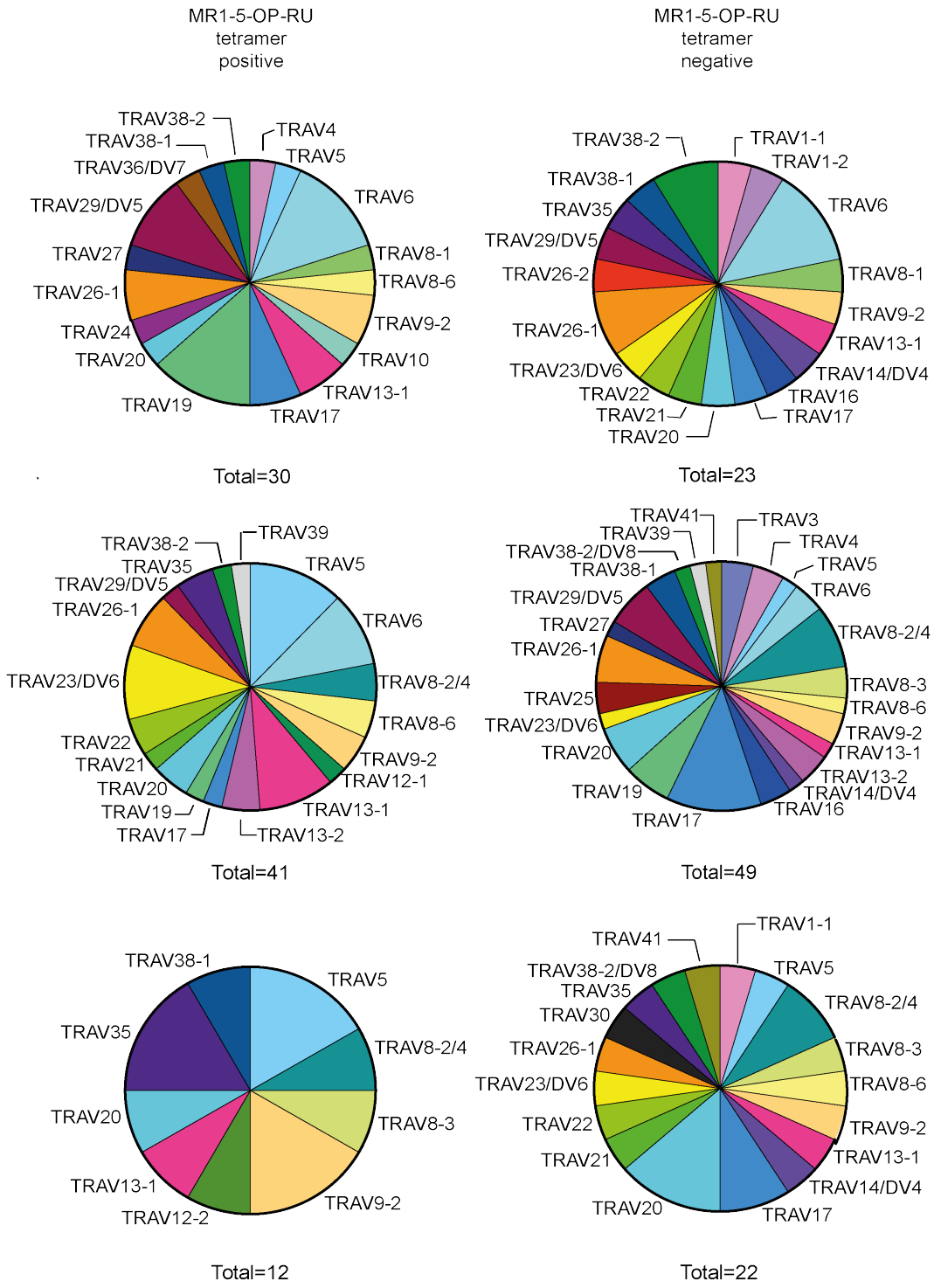


Figure 5. 11 – TRAV gene usage of $CD25^+$ $CD127^+$ $CD4^+$ MR1-5-OP-RU $tetramer^+$ T cells and $CD25^+$ $CD127^+$ $CD4^+$ MR1-5-OP-RU $tetramer^-$ T cells. Human thymocytes were sorted for either $CD3^+CD4^+$ $CD25^+$ $CD127^+$ MR1-5-OP-RU $tetramer^+$ cells or $CD3^+CD4^+$ $CD25^+$ $CD127^+$ MR1-5-OP-RU $tetramer^-$ cells and sequenced for their TCR. The pie charts show the TRAV gene usage for 3 different donors. From each donor MR1-5-OP-RU tetramer positive and neative cells were analyzed. Only complete productive TCRs were analyzed.

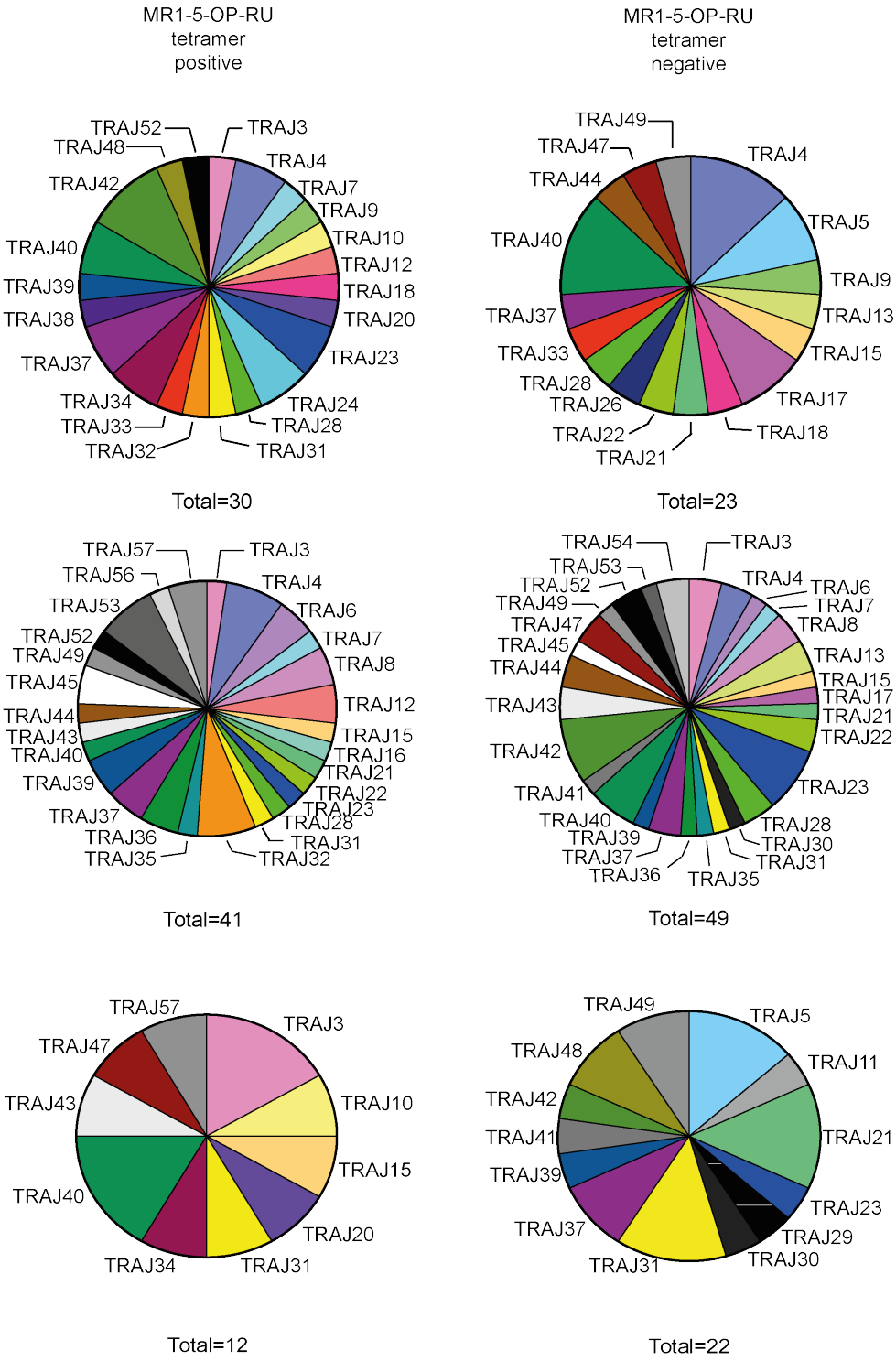


Figure 5. 12 - TRAJ gene usage of $CD25^+$ $CD127^+$ $CD4^+$ MR1-5-OP-RU $tetramer^+$ T cells and $CD25^+$ $CD127^+$ $CD4^+$ MR1-5-OP-RU $tetramer^-$ T cells. Human thymocytes were sorted for either $CD3^+CD4^+$ $CD25^+$ $CD127^+$ MR1-5-OP-RU $tetramer^+$ cells or $CD3^+CD4^+$ $CD25^+$ $CD127^+$ MR1-5-OP-RU $tetramer^-$ cells and sequenced for their TCR. The pie charts show the TRAJ gene usage for 3 different donors. From each donor MR1-5-OP-RU tetramer positive and negative cells were analyzed. Only complete productive TCRs were analyzed.

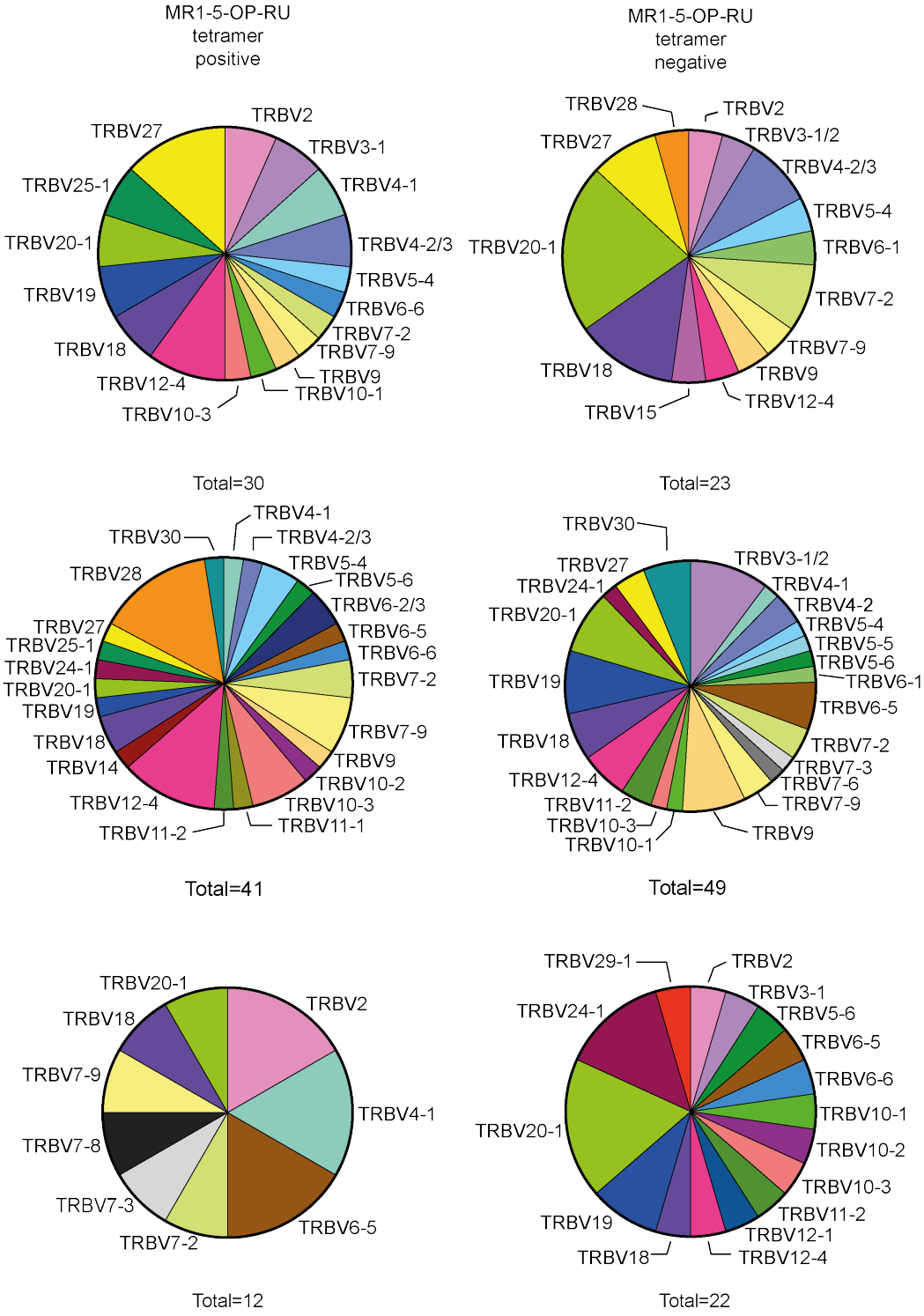


Figure 5. 13 - TRBV gene usage of $CD25^+$ $CD127^+$ $CD4^+$ MR1-5-OP-RU $tetramer^+$ T cells and $CD25^+$ $CD127^+$ $CD4^+$ MR1-5-OP-RU $tetramer^-$ T cells. Human thymocytes were sorted for either $CD3^+CD4^+$ $CD25^+$ $CD127^+$ MR1-5-OP-RU $tetramer^+$ cells or $CD3^+CD4^+$ $CD25^+$ $CD127^+$ MR1-5-OP-RU $tetramer^-$ cells and sequenced for their TCR. The pie charts show the TRBV gene usage for 3 different donors. From each donor MR1-5-OP-RU tetramer positive and negative cells were analyzed. Only complete productive TCRs were analyzed.

5.2.4. MR1 reactivity of specific TCRs

To confirm that the TCRs isolated from the $CD25^+$ $CD127^+$ thymic subset were MR1-reactive, one TCR sequence identified within the MR1-5-OP-RU $tetramer^+$ fraction, and one isolated from MR1-5-OP-RU $tetramer^-$ cells were chosen for transfection experiments. A HEK293T cell line, which does not express a TCR by itself, was transiently transfected with constructs containing the TCR sequences shown in table 5.1. HEK293T cell line was co-transfected with CD3, with or without CD8α, or CD8α and CD8β in order to assess the contribution of these innate-receptors to MR1-recognition. After transfection, cell lines were analyzed using flow cytometry to gauge their reactivity to MR1-5-OP-RU or MR1-6-FP tetramers, with CD1d-α-GalCer tetramers being used as a control.

As shown in figure 5.14 A, total live cells showed MR1-5-OP-RU tetramer staining after transfection with TCR, CD3 and CD8. Cells transfected with CD3/TCR together with CD8 showed high tetramer staining in $CD3^+$ and $CD8^+$ cells, but also low tetramer staining in $CD3^-$ and $CD8^-$ cells. If TCR and CD3 were transfected alone no MR1-5-OP-RU tetramer staining was observed. In contrast, transfection with CD3 and CD8 without any TCR led to MR1-5-OP-RU tetramer staining in $CD8^+$ cells, indicating that CD8 by itself can also bind MR1-tetramers.

For analysis of tetramer mean fluorescence intensity (MFI), cells were gated for $CD3^+CD8^+$ and $CD8^+$ cells with similar expression levels of CD8α, to rule out any effects of CD8 expression levels on MR1-tetramer binding and the MFI (Fig.5.15 A). These two populations ($CD3^+CD8^+$ and $CD8^+$) were then analyzed for the mean fluorescent intensity of MR1 tetramer staining. As shown in figure 5.15 A, cells positive for CD3 and CD8 show a higher MR1-5-OP-RU tetramer staining than $CD8^+$ cells. This indicated that binding of MR1-tetramer is mediated by TCR as well as CD8, since the mean fluorescence intensity of MR1-tetramer staining was enhanced when cells were co-transfected with TCR, suggesting that the

TCR leads to a stronger binding of tetramer. A similar pattern was observed in samples that were additional transfected with CD8β or without a TCR. Quantification of the MFI showed again that cells transfected with CD3/TCR and CD8 and that express these proteins have higher MFI compared to cells from the same well that did not express CD3. Additionally, comparing cells transfected with CD3 and CD8 to cells transfected wit CD3, CD8 and TCR an increased MFI could be observed in cells that are transfected with CD3, CD8 and TCR (Fig.5.15 B). To clarify if this difference is significant an additional experiment needs to be performed. Moreover, as already shown in figure 5.14, cells transfected with CD3 and TCR in the absence of CD8 did not exhibit MR1 tetramer staining regardless of the MR1 ligand (Fig. 5.15 B+D).

These data suggested that the expression of CD8 was required for MR1-tetramer staining and that the TCR/CD3/CD8 complex bound MR1 tetramers in a specific manner. When cells were co-transfected with CD8a and CD8β, a similar staining pattern was observed (Fig. 5.15 C). In contrast to cells transfected with CD8α, the cells transfected with CD8αβ showed a lower MFI of MR1-tetramer staining overall. Comparing cells that were transfected with CD3/CD8/TCR to cells that were only transfected with CD3/CD8αβ no differences were observed, suggesting that TCR expression did not enhance MR1-tetramer binding by CD8αβ. Expression of the TCR sequences isolated from $CD25^+$ $CD127^+$ MR1-$tetramer^-$ cells failed to exhibit MR1-5-OP-RU and MR1-6-FP tetramer staining when they were transfected with CD3 alone (Fig. 5.15 D+E). However, when this TCR was co-transfected with CD8α, a higher MFI was found compared to cells that were transfected with CD8α and CD3 but without TCR similar to the results obtained with the TCR sequence from MR1 $tetramer^+$ cells. This indicated that the TCR sequence in isolation was not able to confer MR1-tetramer binding, but TCR enhance binding of tetramer through CD8. Altogether, this data showed no differences in MR1-tetramer binding between TCRs that were isolated from either MR1-5-OP-RU $tetramer^+$ cells or MR1-5-OP-RU $tetramer^-$ cells, indicating that

the TCR usage does not play a role for MR1-tetramer binding together with CD8 expression. The potential role for CD8 expression in the recognition of MR1 is depicted within figure 5.14 and figure 5.15.

Table 5. 1 - TCRs that were used for the transfection experiment

	TRAV	**TRAJ**	**CDR**	**TRBV**	**TRBJ**	**TRBD**	**CDR**
Tetramer positive	8-6*02	37*02	CAVSGSSNTGKLIF	12-4*02	2-6*01	1*01	CASRHSGANVLTF
Tetramer negative	17*01	43*01	CATDALDNDMRF	9*01	2-4*01	2*01	CASSLGLAGGVAKNIQYF

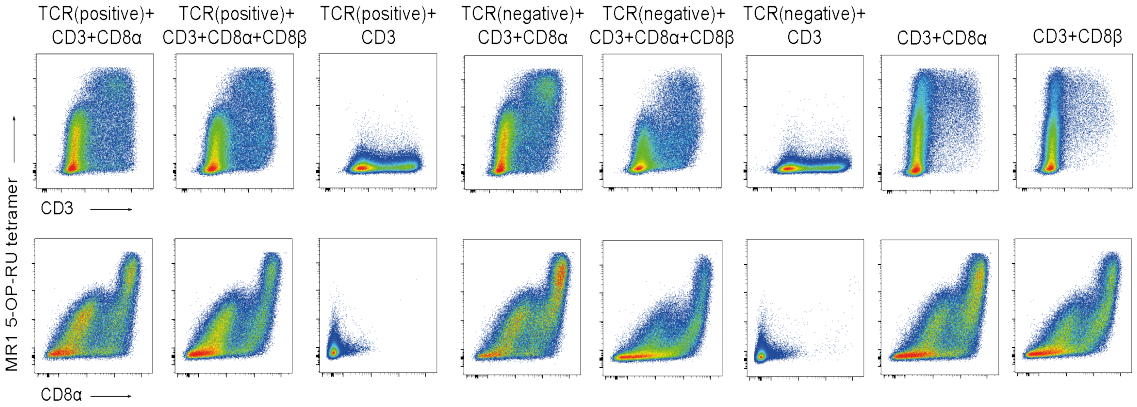


Figure 5. 14 – Representative FACS plots of transiently TCR-transfected HEK293T cell line with different combinations of TCR, CD3 and CD8. The HEK293T cells were transfected with TCR+CD3+CD8, CD3+CD8 or TCR+CD3. After transfection the cells were stained with different tetramers and analyzed using flow cytometry. The plots show MR1-5-OP-RU tetramer staining against CD3 or CD8α on total live cells transfected with different combinations of plasmids.

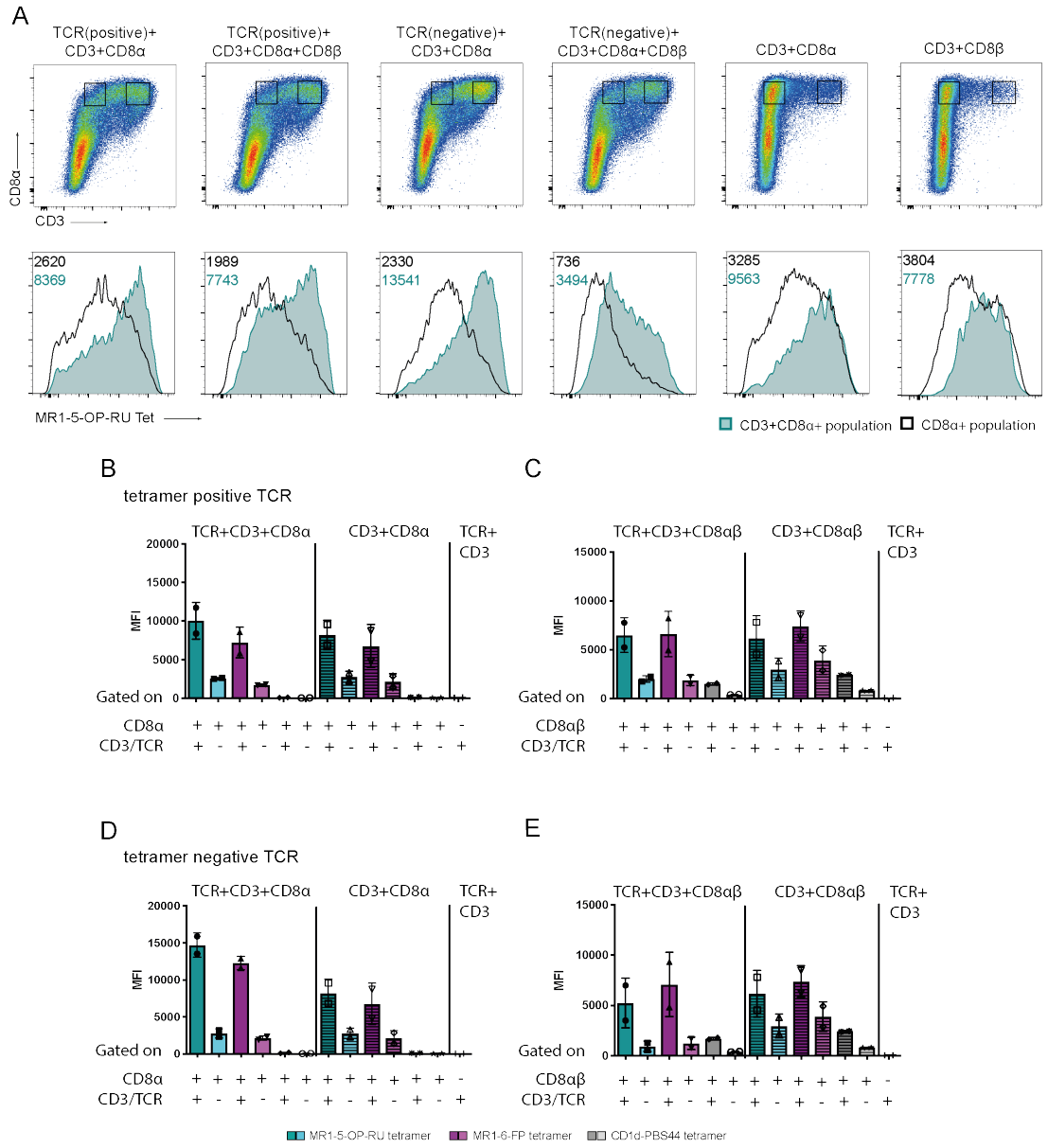


Figure 5. 15 - Mean fluorescence intensity of tetramer staining for different transiently TCR-transfected HEK293T cell line in different combinations of CD3 and CD8. The HEK293T cells were transfected with TCR+CD3+CD8, CD3+CD8 or TCR+CD3. After transfection the cells were stained with different tetramers and analyzed using flow cytometry. The cells were gated on either CD8 or CD3+CD8. Then the mean fluorescence intensity of tetramer staining was analyzed. (A) Representative FACS plots of total live transfected cells with the indicated plasmids showing CD3 against CD8α staining and the gating on $CD3^{+}CD8\alpha^{+}$ cells and $CD3^{-}CD8\alpha^{+}$ cells with similar CD8α expression levels. These two gates were analyzed for MR1-5-OP-RU tetramer staining and the histograms are shown below the dedicated FACS plots. Bar graphs show technical replicates for transfection with tetramer positive TCR + CD8α (B), tetramer positive TCR + CD8α+CD8β (C), tetramer negative TCR + CD8α (D) and tetramer negative TCR +CD8α+CD8β (E). Data represent one experiment.

5.2.5. FOXP3$^+$ T-bet$^+$ MR1-5-OP-RU tetramer$^+$ T cells in PBMCs

The previous results of MR1-reactive cells within the FOXP3$^+$ T-bet+ T population, as well as phenotypic and TCR repertoire analysis were obtained from human thymus samples. To examine whether the FOXP3$^+$ T-bet$^+$ cells that recognize MR1 could be found within the periphery, human PBMCs were isolated from blood and co-stained for FOXP3 and T-bet. After gating on live cells, using 7-amino-actinomycin D (7-AAD), a viability dye, as well as CD14$^-$ and CD19$^-$ cells to exclude monocytes and B cells respectively; T cells were identified using CD3. By using Vα7.2 and MR1-5-OP-RU tetramer, MAIT cells could be clearly identified (Fig. 15.16). By plotting MR1-5-OP-RU tetramer against FoxP3, no cells were identified that bound MR1 tetramer and were positive for FOXP3. Analyzing T-bet expression against MR1 tetramer staining, MR1-5-OP-RU tetramer$^+$ cells can be identified as T-bet intermediate cells, consistent to previous publications (Gherardin *et al.*, 2016; Gherardin *et al.*, 2018). These results indicated that no population of MR1-tetramer$^+$ cells that co-express FOXP3 together with T-bet were identified in human PBMCs. Furthermore, analysis of conventional T cells for co-expression of T-bet and FOXP3 showed no co-expression of FOXP3 and T-bet within CD3$^+$ T cells, indicating that the cells previously described within the thymus cannot be found within human blood, although location in other tissues is possible.

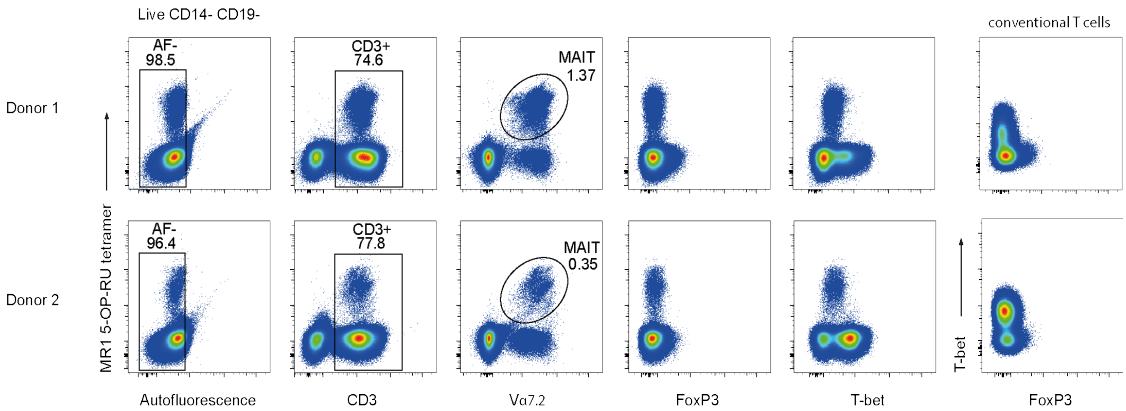


Figure 5. 16 – FOXP3$^+$ T-bet$^+$ T cells in human PBMCs. Representative gating of PBMCs from 2 different donors. First cells were gated on single, live, CD14$^-$ CD19$^-$ cells. They were further gated as autofluorescence- CD3$^+$ cells and were analyzed for staining of MR1-5-OP-RU tetramer co-expressed with Vα7.2, FOXP3 or T-bet. Additionally, conventional T cells (MAIT$^-$ cells) were plotted T-bet againstFOXP3. The data is representative of 2 independent experiments.

5.2.6. FoxP3$^+$ T-bet$^+$ T cells in mice

To investigate if the previously described human FOXP3$^+$ T-bet$^+$ T cell subset can be found in mice, thymus, LNs and the small intestine (SI) of C57BL/6 mice were analyze for their FoxP3 and T-bet expression. Live single cells were gated for CD3 as well as CD4. The CD4$^+$ T cells were then analyzed for FoxP3 and T-bet expression. Around 16 % (LN), 2 % (thymus) and 7.45 % (SI) of T cells are FoxP3$^+$, while 0.4 % (LN), 2 % (thymus) and 75 % (SI) of those cells express T-bet. No clear FoxP3$^+$ T-bet$^+$ population was identified in all three organs (Fig. 5.16). Koch et al. showed that T-bet is upregulated in FoxP3$^+$ T_{regs} upon stimulation and infection, but no FoxP3$^+$ T-bet$^+$ cells were found in the lymph node of naïve mice (Koch *et al.*, 2009). Analyzing MR1-5-OP-RU tetramer staining on the different FoxP3 and T-bet populations, it was shown that MAIT cells which are PLZF positive are mainly found within the FoxP3$^-$ T-bet$^-$ population in the LN because MAIT cells are known to be biased towards RoRγT expression in mice instead of T-bet (Rahimpour *et al.*, 2015). Very little MR1-5-OP-RU tetramer staining was observed within the population

that did not express PLZF (Fig. 15.17 A). Furthermore, very few MR1-5-OP-RU tetramer$^+$ cells were found within the FoxP3$^+$ population in the LN.
In the thymus, some MR1 tetramer$^+$ cells that did not express PLZF were observed within the FoxP3$^-$ T-bet$^-$ population (Fig. 5.17 B), which is consistent to previous findings of stage 1 and stage 2 MAIT cells that do not express PLZF and are T-bet$^-$ in mice. In contrast, the T-bet$^+$ subset showed MR1-5-OP-RU tetramer$^+$ cells that express PLZF in accordance with previous publications (Koay *et al.*, 2016).
In the SI, the FoxP3$^-$ T-bet$^-$ population exhibited MR1-5-OP-RU tetramer staining. The MR1 tetramer$^+$ cells also showed an intermediate PLZF staining and are MAIT cells (Fig. 5.17 C). In general MAIT cells were identified in all analyzed organs with 0.15 % MAIT cells of all live CD19$^-$ cells in the LN, 0.005 % MAIT cells in the thymus and 0.24 % MAIT cells of all live CD19$^-$ cells in the SI. In the lymph node, the majority of MAIT cells were DN (71.5 %), but also CD4$^+$ (23.7 %) or CD8$^+$ (4.28 %) MAIT cells were observed. In the thymus 39.2 % of MAIT cells are DN, 24.6 % are CD8$^+$, while 21.7 % are CD4$^+$ and 14.5 % are DP, consistent with previous studies (Koay *et al.*, 2016). In the SI, 93.1 % of MAIT cells were DN with 6.72 % of CD8$^+$ MAIT cells. All in all, this indicated that FoxP3$^+$ T-bet$^+$ T cells cannot be found in mice. Some MR1 tetramer staining was observed in cells that did not express FoxP3, T-bet and PLZF indicating that maybe other transcription factors expressed in mice by MR1-reactive T cells. Further investigations need to be done to examine the MR1-tetramer$^+$ cells that were displayed here.

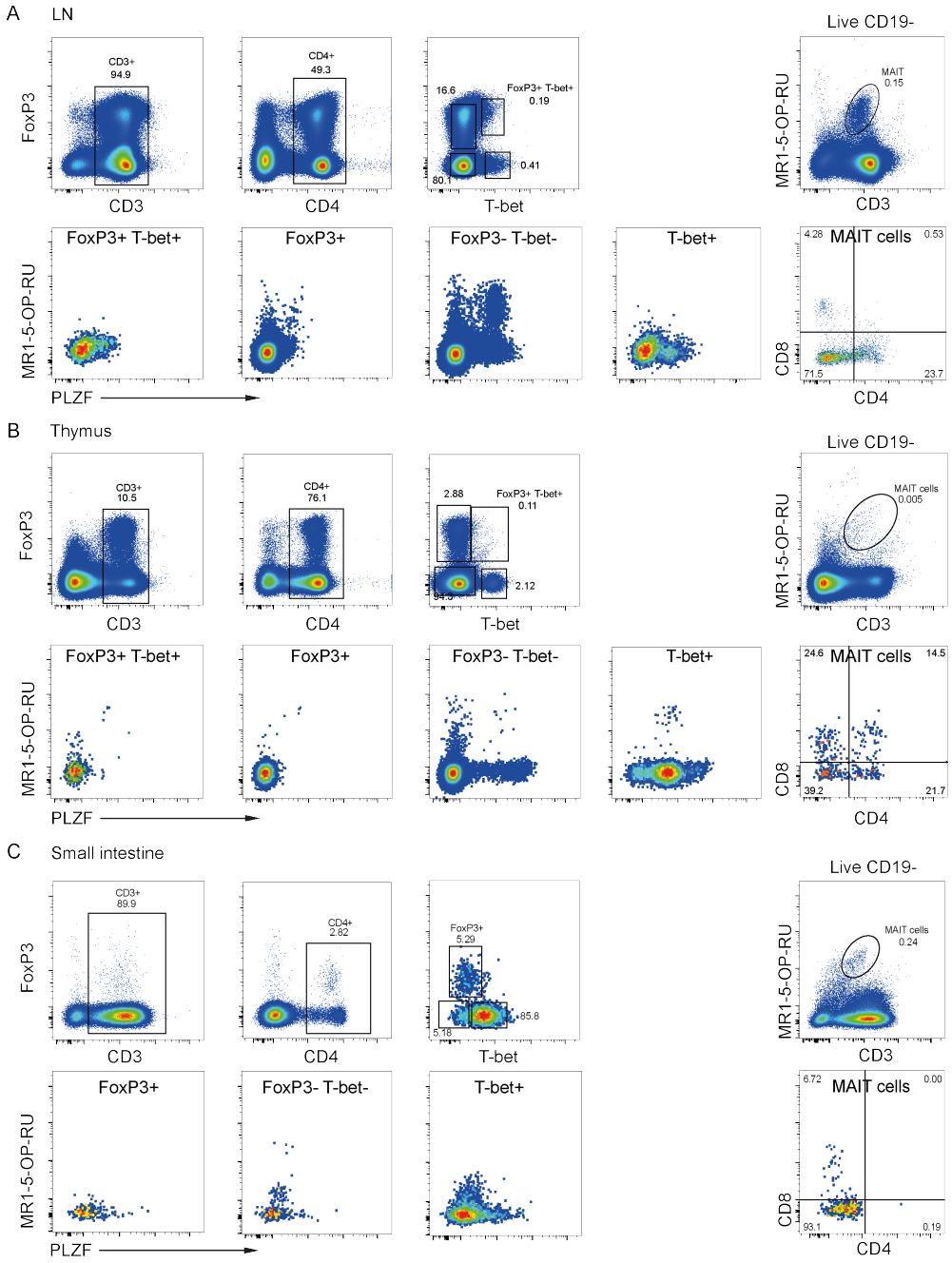


Figure 5. 17 - FoxP3$^+$ T-bet$^+$ MR1 tetramer$^+$ T cells in mice. (A) LNs from mice were gated for CD3$^+$, CD4$^+$ cells and were analyzed for FoxP3 and T-bet expression. The different FoxP3 T-bet populations were analyzed for MR1-5-OP-RU tetramer staining and PLZF expression. Here the representative result from one mouse is shown. (B) Thymus from mice were gated for CD3$^+$, CD4$^+$ cells and were analyzed for FoxP3 and T-bet expression. The different FoxP3 T-bet populations were analyzed for MR1-5-OP-RU tetramer staining and PLZF expression. Here the representative result from one mouse is shown. (C) Small intestine (SI) was gated for CD3$^+$, CD4$^+$ cells and were analyzed for FoxP3 and T-bet expression. The different FoxP3 T-bet populations were analyzed for MR1-5-OP-RU tetramer staining and PLZF expression. Here the representative result from one mouse is shown.

5.3. Discussion

In the present study, the use of MR1 tetramers to identify populations of MR1-reactive cells within the human thymus showed a population of MR1-5-OP-RU tetramer$^+$ T cells. The aim of this chapter is therefore to characterize these cells by expression of immune-relate proteins, as well as TCR usage and verification of MR1 reactivity. Characterization of those MR1-5-OP-RU tetramer$^+$ cells revealed a population of Vα7.2$^-$ cells. Since Vα7.2 is the most commonly variable gene segment expressed by MAIT cells (Tilloy *et al.*, 1999), this indicated that these MR1-tetramer$^+$ cells were not classical MAIT cells, but could be part of previously described atypical MAIT cells (Gherardin *et al.*, 2016). Atypical MAIT cells were described as MAIT cells that are able to bind MR1 tetramer without expression of Vα7.2 (Gherardin *et al.*, 2016). Furthermore, this population of MR1 tetramer$^+$ cells lacked PLZF expression, which is the hallmark of MAIT cell and NKT cell development (Kovalovsky *et al.*, 2008; Savage *et al.*, 2008; Koay *et al.*, 2016).

Further analysis of this subset revealed that MR1 tetramer$^+$ cells co-expressed FOXP3 and T-bet, in contrast to classical MAIT cells that typically express either T-bet or RoRγT. No FOXP3 expression of MAIT cells has been reported so far.

Co-expression of FOXP3 and T-bet is reported in T_{regs} that are able to induce T-bet expression during stimulation in the periphery (Duhen *et al.*, 2012). Furthermore, another study identified T_{regs} that recirculated back to the thymus. This recirculating T_{regs} express T-bet in the thymus (Thiault *et al.*, 2015). FOXP3 as well as T-bet expression can be induced by activation of CD4$^+$ T cells (Matsuoka *et al.*, 2004; Wang *et al.*, 2007).

Besides FOXP3 and T-bet, also CD25 and CD127 were used as surrogate markers to sort the cells for analysis and to perform functional assays. CD25$^+$ CD127$^+$ T cells also displayed recognition of MR1 tetramer, which

correlates with CD8α expression, similar to FOXP3$^+$ T-bet$^+$ T cells. Analyzing the expression of T_{reg} associated proteins, as well as chemokine receptors and T_H1 associated proteins, a similar expression pattern between FOXP3$^+$ T-bet$^+$ and CD25$^+$ CD127$^+$ cells was observed for the analyzed proteins. This indicated that FOXP3$^+$ T-bet$^+$ T cells have a similar phenotype as CD25$^+$ CD127$^+$ T cells. Furthermore, co-staining of FOXP3, T-bet, CD25 and CD127, indicated that ¾ of FOXP3$^+$ T-bet$^+$ T cells were located within the CD25$^+$ CD127$^+$ T cell gate. The same was discovered when CD25$^+$ CD127$^+$ T cells were analyzed for FOXP3 and T-bet expression. Around 75 % of CD25$^+$ CD127$^+$ T cells were found to be FOXP3$^+$ T-bet$^+$. This suggests that CD25 and CD127 can be used as surrogate markers for FOXP3 and T-bet and that the same cells can be identified using either FOXP3/T-bet or CD25/CD127.

Phenotypic analysis of FOXP3$^+$ T-bet$^+$ T cells or CD25$^+$ CD127$^+$ T cells showed a clear expression of T_{reg} associated proteins compared to the other populations (FOXP3$^-$ T-bet$^-$; FOXP3$^+$ T-bet$^-$; FOXP3$^-$ T-bet$^+$ and MAIT cells). Especially CTLA-4 and ICOS were expressed in high levels on FOXP3$^+$ T-bet$^+$ T cells. In contrast, GITR only showed a shift in expression, which could be explained due to a antibody with low affinity, since also T_{regs} should express GITR within thymus. Contrary to FOXP3$^+$ T-bet$^+$ T cells, FOXP3$^+$ T cells are mainly ICOS$^-$, consistent to published studies where newly developed T_{regs} did not express ICOS (Thiault *et al.*, 2015).

Moreover, no CD103 expression was observed on FOXP3$^+$ T-bet$^+$ cells. Also FOXP3$^+$ T cells did not express CD103, even though it is known to be expressed during T_{reg} development (Nunes-Cabaço *et al.*, 2011). Analyzing all CD3$^+$ T cells, CD103 expression was observed on CD8$^+$ T cells in the thymus, confirming that the antibody is working. Furthermore, a small percentage of MAIT cells expressed CD103. These CD103$^+$ MAIT cells could represent mature MAIT cells, under the condition that they are behaving like murine immature MAIT cells that are CD103$^-$ (Koay *et al.*,

2016). This could indicate FOXP3$^+$ T-bet$^+$ T cells are immature. Besides T_{reg} associated proteins, also T_H1 associated proteins, like IL-18R (Chan *et al.*, 2001) were analyzed. IL-18R and CD212 are also proteins that are expressed by MAIT cells, while in the thymus the expression of IL-18R is restricted to stage 3 MAIT cells. FOXP3$^+$ T-bet$^+$ cells expressed IL-18R as well as CD212. This could indicate that they are mature cells, even though that would stand in contrast to their CD103 expression. Besides IL-18R and CD212, FOXP3$^+$ T-bet$^+$ cells expressed CXCR3. T_H1 cells normally express CXCR3, regulated by T-bet expression (Sallusto, Lenig, *et al.*, 1998; Bonecchi *et al.*, 1998; Lord *et al.*, 2005). But recently a study identified CXCR3$^+$ T_{regs} in mice. These CXCR3$^+$ T_{regs} also express T-bet upon infections, but not in naïve mice (Koch *et al.*, 2009). Altogether, FOXP3$^+$ T-bet$^+$ T cells show a T_H1/T_{reg} phenotype that is different to T_{regs}, T_H1 cells and MAIT cells.

Even though the phenotype and TCR usage of CD25$^+$ CD127$^+$ T cells were clarified, some questions still remain open. One question is whether FOXP3$^+$ T-bet$^+$ T cells have suppressive capacity like normal T_{regs}. Preliminary data already showed that CD25$^+$ CD127$^+$ T cells produce IL-8, IL-10 as well as IFNγ, indicating that CD25$^+$ CD127$^+$ cells produce cytokines correlated with T_{regs} (Himmel *et al.*, 2011).

Another important finding in this study is that the MR1 tetramer staining on FOXP3$^+$ T-bet$^+$ T cells correlated with CD8α expression levels. It has been established recently within our laboratory that CD8αα homodimer and CD8αβ heterodimer are ligands for MR1 (unpublished data, Michael Souter). Data presented herein also showed that CD8α and CD8αβ are ligands for MR1. The data of TCR transduced-cell line also indicated that CD8 is important for binding of MR1 tetramers to the TCRs from CD25$^+$ CD127$^+$ cells. Expression of TCR together with CD3 and CD8 led to MR1 tetramer staining. Surprisingly, also MR1 tetramer staining was observed in samples where no TCR but CD3 and CD8 were transfected.

This could indicate that CD8 is the ligand for tetramer here, independent of the TCR expressed. However, addition of TCR enhanced the MR1 tetramer staining, indicating that both CD8 and TCR are important for binding of MR1 tetramers. In the experimental setup used, CD8 expression levels that resulted from transient transfections were unphysiologically high. These unphysiological levels of CD8 could lead to MR1 tetramer binding without TCR. In a setting with lower CD8 levels MR1 tetramer binding could appear only when TCR and CD8 are present, while low CD8 levels could not lead to MR1-tetramer binding by itself. Therefore the experiment definitely needs to be repeated with a more physiological expression of CD8 to rule out that CD8 is the only mediator of MR1 tetramer binding of $CD25^+$ $CD127^+$ T cells.

Despite clear and repeatable identification of FOXP3+ T-bet+ T cells within the human thymus, no $FOXP3^+T\text{-}bet^+$ cells were identified within PBMCs. This could be due to the fact that the cells in the thymus are still in development and change their transcription factor profile upon thymic egress. Furthermore, they may home to certain tissues, thus being difficult to detect within the blood. One place where those cells could be found is the intestine. It is known that $CD4^+$ T cells can upregulate CD8α and T-bet in response to the intestinal environment (Reis *et al.*, 2014). It was shown that especially microbial tryptophan metabolites can upregulate CD8α on $CD4^+$ T cells (Cervantes-Barragan *et al.*, 2017). The identified cells could egress the thymus as $CD4^+$ T cells that regain CD8α expression in the periphery. Interestingly, $FoxP3^+$ $T\text{-}bet^+$ T cells did not express any gut-homing molecules, like CD103 or CCR9, in the thymus, which could be due to the fact that they still immature T cells. Besides this, it is also described in mice that $CD103^-$ T cells in thymus give rise to IELs (Guo *et al.*, 2015).

Analysis of FoxP3 and T-bet expression in mouse lymphocytes failed to identify a clear population of cells that co-expressed these two

transcription factors. Additionally, no MR1 tetramer staining was observed in cells that singularly expressed FoxP3 or T-bet. One explanation for no identification of FoxP3$^+$ T-bet$^+$ cells could be the use of other transcription factors than FoxP3 and T-bet in MR1-reactive T cells in mice. Interestingly, in mice MAIT cells are mainly DN, while in humans most MAIT cells are CD8$^+$ (Reantragoon *et al.*, 2013; Rahimpour *et al.*, 2015). It has yet to be established if CD8 is a co-receptor for MR1 in mice too. If CD8 could not bind to MR1 in mice, this would explain why no MR1 tetramer staining was observed within FoxP3 or T-bet$^+$ cells.

Altogether, these results indicate that there are undescribed FoxP3$^+$ T-bet$^+$ T cells within human thymus that are able to bind to MR1 tetramers. They displayed a T_{reg}/T_H1 phenotype and had a diverse usage of TCR. The question if these cells are progenitors for T_{regs}, IELs or other T cells or if they are a new population of MR1-reactive T cells still needs to be investigated. All in all, these data demonstrate that the scope of immune cells capable of interacting with MR1 may extend beyond our current understanding.

6. Chapter 6: Overall Discussion

MAIT cells are unconventional T cells with an invariant TCR α-chain that is not donor dependent like for MHC restricted conventional T cells. Furthermore, they recognize metabolites presented by the non-polymorphic protein MR1 that is ubiquitously expressed. Together with their feature to migrate into tissues this makes them good targets for immunotherapeutic therapies. So far MAIT cells and their functions were extensively studied during bacterial infections because of microbial metabolite recognition (Kjer-Nielsen *et al.*, 2012). Upon activation, MAIT cells produce high amounts of IFNγ, IL-17 and TNFα, indicating a proinflammatory role of MAIT cells. Interestingly, some recent studies analyzed MAIT cells in autoimmune diseases and suggested a protective role of MAIT cells for example in multiple sclerosis (Croxford *et al.*, 2006; Miyazaki *et al.*, 2011). Therefore, it is important to investigate the role of MAIT cells within the immune system.

In chapter 3, it was shown that MAIT cells could induce DC migration and maturation. The co-stimulatory molecules CD86, CD80 and CD40 were upregulated upon stimulation of MAIT cells with high dose of activating MAIT cell antigen, while CD80 and CD40 expression remained unchanged after MAIT cell activation with low dose antigen. These results suggest that MAIT cells could induce maturation of DCs dependent on antigen availability. Low dose antigen could lead to a tolerogenic immune response since only CD86 is upregulated in the medLN, while expression of CD80 and CD40 is unchanged. Here the amount of antigen could not be sufficient to induce a full maturation of DCs. In contrast, high dose of antigen could induce full maturation of DCs. Of course, the results in this thesis are not complete at this stage. Furthermore, the induction of a tolerogenic response by low dose antigen needs to be proved. Therefore the cytokine production by DCs as well as their transcriptome could be analyzed to compare them to DCs that were stimulated with TLR ligands and that are able to induce an immunogenic immune response. This could clarify what phenotype DCs

have after stimulation by MAIT cells and if this phenotype differs to TLR-maturated DCs. Furthermore, induction of T_{regs} by these DCs could be analyzed.

Besides co-stimulatory molecules, chemokine expression by DCs was induced upon MAIT cell activation. In chapter 3, MAIT cell activation was shown to induce the production of CCL17 as well as CCL22 by DCs. The production of those two chemokines by DCs is also induced by NKT cell-mediated DC activation (Semmling *et al.*, 2010). The similarities between MAIT and NKT cells could indicate that MAIT cells activate DCs in a similar fashion as NKT cells. The production of CCL17 and CCL22 induced by NKT cells is important for the recruitment of $CCR4^+$ CTLs leading to efficient $CD8^+$ T cell priming. CCL17 as well as CCL22 production induced by MAIT cells could indicate that DCs recruit $CCR4^+$ CTLs for inducing a $CD8^+$ T cell response. In chapter 4, no enhanced $CD8^+$ T cell priming was observed by MAIT cell activation, indicating that MAIT cells do not induce similar immune responses as NKT cells. Besides the recruitment of $CD8^+$ T cells, attraction of other $CCR4^+$ cells is possible by CCL17 and CCL22. CCR4 is expressed by T_{regs} as well as T_H2 cells (Imai *et al.*, 1999; Campbell *et al.*, 1999; Iellem *et al.*, 2001), which could indicate that MAIT cells activation could lead to recruitment of regulatory cells. To clarify this hypothesis, further investigations need to be done, especially if and what types of cells are recruited by CCL17 and CCL22 production after MAIT cell activation. To test this, CCL17 knock out mice could be used and changes in the immune cell composition upon MAIT cell activation could be analyzed.

In general, the question of how MAIT cells can influence the immune response by impacting on DCs and if this impact is similar or distinct to NKT cells arise. With the results obtained in this thesis, MAIT cells seem to induce a distinct immune response compared to NKT cells. No enhancement of $CD8^+$ T cell priming was observed by harnessing MAIT cells, even though some results like co-stimulatory molecules and chemokine production could indicate an influence on CTL priming. Further

experiments with higher doses of 5-A-RU/MeG for vaccinations need to be performed to clarify the effects of MAIT cells on $CD8^+$ T cells.

Another important finding observed in chapter 3, is the production of the cytokines IL-13, IL-10, IL-4, GM-CSF as well as IL-6 by MAIT cells. IL-13, IL-4 and IL-10 can suppress cell mediated immune responses and could indicate an inhibitory role for MAIT cells, but it has to be validated if the observed cytokine production can be found *in vivo* as well, since the results were obtained during *in vitro* stimulation assays.

The cytokines IL-13 and IL-4 are associated with immune suppression in tumor microenvironments (Terabe *et al.*, 2004; Rawal *et al.*, 2011). This could indicate that MAIT cells play an important role in cancer. By producing these cytokines MAIT cells could suppress anti-tumor immune responses leading to tumor progression. The knowledge that MAIT cells can induce immune suppression could open up a new field of possible treatments. Harnessing MAIT cells in a way that they switch to pro-inflammatory cytokine production is just one possibility.

Besides cancer, it was shown that the cytokines IL-13 and IL-4 are associated with asthma induction (Doran *et al.*, 2017), indicating that also here MAIT cells could play an important role in this disease. A few studies suggest a protective role of MAIT cells in asthma, since lower MAIT cell number correlate with severity (Hinks *et al.*, 2015) and higher MAIT cell frequencies in one-year old children were associated with decreased risk of asthma development (Chandra *et al.*, 2018). The role of MAIT cells in asthma needs further studies. Clarification if MAIT cells play a more protective or negative role is important, especially with the now identified production of IL-13. Here MAIT cells could be a source of IL-13 in asthmatic lungs, since MAIT cells are abundant in lung tissue. The produced IL-13 could lead to more serve disease. Therefore it is important to study, whether MAIT cells contribute in a positive or negative way to asthma. Clarity about their role could lead to development of new therapeutic therapies by harnessing MAIT cells.

Besides a suppressive function of MAIT cells, therapeutic vaccination by harnessing MAIT cells could be of great interest. NKT cells were already harnessed for vaccinations since they induce antigen-specific CTL responses after co-injecting αGalCer and antigens. Furthermore, injection of αGalCer with tumor-specific antigen induced anti-tumor responses, indicating that NKT cells could be a target for vaccinations (Ian F. Hermans *et al.*, 2003; Cerundolo *et al.*, 2009).

MAIT cells share features with NKT cells, which suggests that they can have similar functions and can be used in a similar way in immunotherapeutic therapies. Therefore it is important to test, whether MAIT cells can also be harnessed for vaccination strategies. Targeting MAIT cells could be more promising in contrast to NKT cells, since they are more frequent in humans than NKT cells (Godfrey *et al.*, 2015). Therefore, an important question is the understanding of the role of MAIT cells in the immune system and interactions with other immune cells, especially the impact of MAIT cells on DCs and their ability to induce CTL responses are important to study. As already described, MAIT cells can activate DCs, but if this activation is sufficient to induce an antigen-specific T cell response, was analyzed in chapter 4.

No enhanced antigen-specific $CD8^+$ T cell responses after MAIT cell activation were observed with low dose antigen, indicating that MAIT cells are not able to induce complete maturation of DCs, but more an incomplete maturation in this setting, where DCs are not able to induce an immunogenic immune response. Here it is important to also test these vaccination strategies with high dose antigen to clarify if full maturation of DCs can induce immunogenic $CD8^+$ T cell response. Furthermore, the expression of suppressive cytokines by MAIT cells could explain the absence of an immunogenic CTL response after MAIT cell activation observed in chapter 4. Another possibility is that MAIT cells prevent an immunogenic T cell response by promoting a tolerized DC phenotype, while they start to induce an immunogenic response, when co-stimulation is present for example in

the presence of a TLR ligand. It was shown *in vivo* that the presence of TLR ligands is important for MAIT cell proliferation (Chen *et al.*, 2016). This could indicate that microbial metabolic antigens activate MAIT cells, but activation does not reach a specific threshold leading only to maturation of DCs, but no T cell priming. Important factors for priming could be missing by MAIT cell activation with microbial antigens only. By adding a TLR ligand, the activation of MAIT cells could be higher leading to proliferation and reaching a threshold where MAIT cells can induce full DC maturation and effective CTL responses. This suggests that MAIT cells are part of a complex system with suppressive and activating signals that are counterbalanced to prevent permanent activation of MAIT cells by commensal bacteria. Only the presence of other stimuli leads to a full activation. To test this hypothesis, further investigations are needed. By using different concentrations of TLR ligand during vaccination as well as different MAIT cell antigen concentrations, an immunogenic $CD8^+$ T cell response could be induced that is promoted by MAIT cells. If MAIT cells could enhance a $CD8^+$ T cell response, this would open up a whole new field of possible immunotherapeutic therapies. Since NKT cells are less frequent in humans than MAIT cells (Godfrey *et al.*, 2015), this could lead to a valuable new approach in vaccine development, because more donor-independent cells could react to vaccination.

All together, this indicates that MAIT cells are potentially able to modulate the immune response beyond their immediate effector function. So far the results suggest that MAIT cells can act on DCs, but more investigations need to be done, to clarify the impact of MAIT cells on autoimmune diseases and antigen-specific $CD8^+$ T cells, since this will have a great impact on vaccination research and the role of MAIT cells in the immune system.

Besides the effect of MAIT cells on the immune system, MR1-reactive T cells were analyzed in human thymus. Thereby, cells were found that bound MR1 tetramers, but showed a distinct phenotype to MAIT cells. These

T cells were identified as part of a FOXP3$^+$ T-bet$^+$ T cell population. Since their phenotype was clearly distinguishable from classical MAIT cells, these cells are maybe part of atypical MR1-restricted T cells that were previously described (Gherardin *et al.*, 2016; Koay *et al.*, 2019). The cells identified here could be progenitors of MR1-restricted T cells as well as T_{regs}, since FOXP3$^+$ T-bet$^+$ T cells showed a high expression of T_{reg} markers. However it could also be possible that the cells identified here are an undescribed T cell population. Therefore, further investigations need to be done, to clarify the origin of these cells. Transcriptomic analysis could be performed to analyze and compare the cells to T_{regs}, T_{reg} progenitors and MAIT cells during their different developmental stages on basis of RNA transcripts. Besides their origin, it is also important to know if the cells are present in the periphery. In chapter 5, the cells could not be identified in human PBMCs by usage of the markers that were used for identification in human thymus. The markers used for identification in human thymus could be different after the cells egress from thymus, making it difficult to track them in the periphery. One useful tool would be to identify the cells in mice where they can be easily manipulated to investigate their origin, function and location. In chapter 5, the identification of the cells in different murine tissues was tried, but no clear population of FoxP3$^+$ T-bet$^+$ MR1-reactive T cells were found. This could be due to different usage of markers between human and mice, since also MAIT cells differ between the two species.

Furthermore, it is important to analyze the functions of those cells. As shown in chapter 5 the cells have a T_{reg} like phenotype suggesting that they have regulatory potential. Therefore it is important to investigate if they have the ability to suppress for example T cell expansion. Co-culturing FOXP3$^+$ T-bet$^+$ T and CD8$^+$ T cells during CD3/CD28 stimulation could clarify if FOXP3$^+$ T-bet$^+$ T cells suppress CD8$^+$ T cell proliferation.

Interestingly, FOXP3$^+$ T-bet$^+$ cells bound MR1 tetramers in correlation to CD8α expression. Speculating that FOXP3$^+$ T-bet$^+$ MR1 tetramer$^+$ T cells downregulate CD8α upon development, this could explain why no FOXP3$^+$ T-bet$^+$ MR1 tetramer$^+$ cells were found in human PBMCs. It is known that

$CD4^+$ T cells could upregulate CD8α upon entering the gut due to special stimulatory molecules, like tryptophan metabolites from bacteria (Cervantes-Barragan *et al.*, 2017). A still open question in MAIT cell research is how MAIT cells are regulated in mucosal sites like the gut where riboflavin-producing bacteria are present in the microflora. These bacteria do not activate MAIT cells and therefore the regulation of MAIT cells at mucosal sites is still an unclear process and the presence of a regulatory cell that inhibit the MAIT cell activation is likely. The cells identified in chapter 5 could enter the gut leading to upregulation of CD8α in a bacteria-induced manner, leading to MR1-recognition and regulation due to their FOXP3 expression that is correlated with regulatory functions. This hypothesis is based on the results presented in chapter 5, and these results could mark the start of investigating regulatory MR1-reactive T cells. The question of whether regulatory MAIT cells exist is very important and is asked for many years now. By the use of MR1 tetramers, it should be possible to screen human gastrointestinal tract samples for MR1-reactivity and the presence of regulatory MR1-reactive T cells. Therefore, human gastrointestinal tract samples could be analyzed for MR1 tetramer staining together with expression of FOXP3 via flow cytometry or microscopy. Furthermore, MR1 tetramer$^+$ T cells could be isolated and cultured together with MAIT cells upon antigen stimulation, followed by analysis of MAIT cell activation, which should be lowered upon presence of suppressive cells.

All together, this thesis serves as a basis for further investigations on MR1-reactive T cells and could unravel new subpopulations of MAIT cells or MR1-reactive T cells. Furthermore, it highlights possible suppressive functions of MAIT cells and MR1-reactive T cells.

7. References

Akbari, O., Panjwani, N., Garcia, S., Tascon, R., Lowrie, D. and Stockinger, B. (1999) 'DNA Vaccination: Transfection and Activation of Dendritic Cells as Key Events for Immunity'. *Journal of Experimental Medicine*, 189(1), pp. 169–178. DOI: 10.1084/jem.189.1.169.

Alexopoulou, L., Holt, A.C., Medzhitov, R. and Flavell, R.A. (2001) 'Recognition of Double-Stranded RNA and Activation of NF-KB by Toll-like Receptor 3'. *Nature*, 413(6857), pp. 732–738. DOI: 10.1038/35099560.

Alferink, J., Lieberam, I., Reindl, W., Behrens, A., Weiß, S., Hüser, N., Gerauer, K., Ross, R., Reske-Kunz, A.B., Ahmad-Nejad, P., Wagner, H. and Förster, I. (2003) 'Compartmentalized Production of CCL17 in Vivo: Strong Inducibility in Peripheral Dendritic Cells Contrasts Selective Absence from the Spleen'. *Journal of Experimental Medicine*, 197(5), pp. 585–599. DOI: 10.1084/jem.20021859.

Allan, R.S., Waithman, J., Bedoui, S., Jones, C.M., Villadangos, J.A., Zhan, Y., Lew, A.M., Shortman, K., Heath, W.R. and Carbone, F.R. (2006) 'Migratory Dendritic Cells Transfer Antigen to a Lymph Node-Resident Dendritic Cell Population for Efficient CTL Priming'. *Immunity*, 25(1), pp. 153–162. DOI: 10.1016/j.immuni.2006.04.017.

Amarante-Mendes, G.P., Adjemian, S., Branco, L.M., Zanetti, L.C., Weinlich, R. and Bortoluci, K.R. (2018) DOI: 10.3389/fimmu.2018.02379.

Andersen, M.H., Schrama, D., thor Straten, P. and Becker, J.C. (2006) 'Cytotoxic T Cells'. *Journal of Investigative Dermatology*, 126(1), pp. 32–41. DOI: 10.1038/sj.jid5700001.

Annunziato, F., Cosmi, L., Liotta, F., Lazzeri, E., Manetti, R., Vanini, V., Romagnani, P., Maggi, E. and Romagnani, S. (2002) 'Phenotype, Localization, and Mechanism of Suppression of CD4+CD25+ Human Thymocytes'. *Journal of Experimental Medicine*, 196(3), pp. 379–387. DOI: 10.1084/jem.20020110.

Armbruster, N.S., Richardson, J.R., Schreiner, J., Klenk, J., Günter, M. and Autenrieth, S.E. (2016) 'Staphylococcus Aureus PSM Peptides Induce Tolerogenic Dendritic Cells upon Treatment with Ligands of Extracellular and Intracellular TLRs'. *International Journal of Medical Microbiology*, 306(8), pp. 666–674. DOI: 10.1016/j.ijmm.2016.09.002.

Bachmann, M.F., Kopf, M. and Marsland, B.J. (2006) 'Opinion: Chemokines: More than Just Road Signs'. *Nature Reviews Immunology*, 6(2), pp. 159–164. DOI: 10.1038/nri1776.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.-J., Pulendran, B. and Palucka, K. (2000) 'Immunobiology of Dendritic Cells'. *Article in Annual Review of Immunology*, 18, pp. 767–811. DOI: 10.1146/annurev.immunol.18.1.767.

Bao, K. and Reinhardt, R.L. (2015) 'The Differential Expression of IL-4 and IL-13 and Its Impact on Type-2 Immunity'. *Cytokine*, 75(1), pp. 25–37. DOI: 10.1016/j.cyto.2015.05.008.

Bennett, M.S., Trivedi, S., Iyer, A.S., Hale, J.S. and Leung, D.T. (2017) 'Human Mucosal-Associated Invariant T (MAIT) Cells Possess Capacity for B Cell Help'. *Journal of Leukocyte Biology*, 102(5), pp. 1261–1269. DOI: 10.1189/jlb.4a0317-116r.

Bennett, S.R., Carbone, F.R., Karamalis, F., Flavell, R.A., Miller, J.F. and Heath, W.R. (1998) 'Help for Cytotoxic-T-Cell Responses Is Mediated by CD40 Signalling.' *Nature*, 393(6684), pp. 478–80. DOI: 10.1038/30996.

Bennett, S.R., Carbone, F.R., Karamalis, F., Miller, J.F. and Heath, W.R. (1997) ‘Induction of a CD8+ Cytotoxic T Lymphocyte Response by Cross-Priming Requires Cognate CD4+ T Cell Help.’ *The Journal of Experimental Medicine*, 186(1), pp. 65–70. DOI: 10.1084/jem.186.1.65.

Bevan, M.J. (1976) ‘Cross-Priming for a Secondary Cytotoxic Response to Minor H Antigens with H-2 Congenic Cells Which Do Not Cross-React in the Cytotoxic Assay.’ *The Journal of Experimental Medicine*, 143(5), pp. 1283–1288. DOI: 10.1084/jem.143.5.1283.

Bonecchi, R., Bianchi, G., Bordignon, P.P., D’Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P.A., Mantovani, A. and Sinigaglia, F. (1998) ‘Differential Expression of Chemokine Receptors and Chemotactic Responsiveness of Type 1 T Helper Cells (Th1s) and Th2s’. *Journal of Experimental Medicine*, 187(1), pp. 129–134. DOI: 10.1084/jem.187.1.129.

Le Bourhis, L., Martin, E., Péguillet, I., Guihot, A., Froux, N., Coré, M., Lévy, E., Dusseaux, M., Meyssonnier, V., Premel, V., Ngo, C., Riteau, B., Duban, L., Robert, D., Huang, S., Rottman, M., Soudais, C. and Lantz, O. (2010) ‘Antimicrobial Activity of Mucosal-Associated Invariant T Cells.’ *Nature Immunology*, 11(8), pp. 701–708. DOI: 10.1038/ni1010-969a.

Bousso, P. and Albert, M.L. (2010) ‘Signal 0 for Guided Priming of CTLs: NKT Cells Do It Too.’ *Nature Immunology*, 11(4), pp. 284–286. DOI: 10.1038/ni0410-284.

Bretscher, P. and Cohn, M. (1970) ‘A Theory of Self-Nonself Discrimination’. *Science*, 169(3950), pp. 1042–1049. DOI: 10.1126/science.169.3950.1042.

Brozova, J., Karlova, I. and Novak, J. (2016) ‘Analysis of the Phenotype and Function of the Subpopulations of Mucosal-Associated Invariant T Cells’. *Scandinavian Journal of Immunology*, 84(4), pp. 245–251. DOI: 10.1111/sji.12467.

Campbell, J.J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D.P., Warnke, R., Ruffing, N., Kassam, N., Wu, L. and Butcher, E.C. (1999) 'The Chemokine Receptor CCR4 in Vascular Recognition by Cutaneous but Not Intestinal Memory T Cells'. *Nature*, 400(6746), pp. 776–780. DOI: 10.1038/23495.

Campbell, J.J. and Butcher, E.C. (2000) 'Chemokines in Tissue-Specific and Microenvironment-Specific Lymphocyte Homing'. *Current Opinion in Immunology*, 12(3), pp. 336–341. DOI: 10.1016/S0952-7915(00)00096-0.

Carbone, F.R., Kurts, C., Bennett, S.R.M., Miller, J.F.A.P. and Heath, W.R. (1998) DOI: 10.1016/S0167-5699(98)01301-2.

Carbone, F.R. and Bevan, M.J. (1990) 'Class I-Restricted Processing and Presentation of Exogenous Cell-Associated Antigen in Vivo'. *Journal of Experimental Medicine*, 171(2), pp. 377–387. DOI: 10.1084/jem.171.2.377.

Carrette, F. and Surh, C.D. (2012) 'IL-7 Signaling and CD127 Receptor Regulation in the Control of T Cell Homeostasis'. *Seminars in Immunology*, 24(3), pp. 209–217. DOI: 10.1016/j.smim.2012.04.010.

Castellino, F., Huang, A.Y., Altan-Bonnet, G., Stoll, S., Scheinecker, C. and Germain, R.N. (2006) 'Chemokines Enhance Immunity by Guiding Naive CD8+ T Cells to Sites of CD4+ T Cell-Dendritic Cell Interaction.' *Nature*, 440(7086), pp. 890–895. DOI: 10.1038/nature04651.

Cerundolo, V., Silk, J.D., Masri, S.H. and Salio, M. (2009) 'Harnessing Invariant NKT Cells in Vaccination Strategies'. *Nature Reviews Immunology*, 9(1), pp. 28–38. DOI: 10.1038/nri2451.

Cervantes-Barragan, L., Chai, J.N., Tianero, M.D., Di Luccia, B., Ahern, P.P., Merriman, J., Cortez, V.S., Caparon, M.G., Donia, M.S., Gilfillan, S., Cella, M., Gordon, J.I., Hsieh, C.S. and Colonna, M. (2017) 'Lactobacillus Reuteri Induces Gut Intraepithelial CD4+CD8αα+ T Cells'. *Science*, 357(6353), pp. 806–810. DOI: 10.1126/science.aah5825.

Chan, W.L., Pejnovic, N., Lee, C.A. and Al-Ali, N.A. (2001) 'Human IL-18 Receptor and ST2L Are Stable and Selective Markers for the Respective Type 1 and Type 2 Circulating Lymphocytes'. *The Journal of Immunology*, 167(3), pp. 1238–1244. DOI: 10.4049/jimmunol.167.3.1238.

Chandra, S., Wingender, G., Greenbaum, J.A., Khurana, A., Gholami, A.M., Ganesan, A.-P., Rosenbach, M., Jaffee, K., Gern, J.E., Wood, R., O'Connor, G., Sandel, M., Kattan, M., Bacharier, L., Togias, A., Horner, A.A. and Kronenberg, M. (2018) 'Development of Asthma in Inner-City Children: Possible Roles of MAIT Cells and Variation in the Home Environment'. *Journal of Immunology (Baltimore, Md.: 1950)*, 200(6), pp. 1995–2003. DOI: 10.4049/jimmunol.1701525.

Chen, W.J., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G. and Wahl, S.M. (2003) 'Conversion of Peripheral CD4 + CD25 - Naive T Cells to CD4 + CD25 + Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3'. *Journal of Experimental Medicine*, 198(12), pp. 1875–1886. DOI: 10.1084/jem.20030152.

Chen, Z., Wang, H., D'Souza, C., Sun, S., Kostenko, L., Eckle, S.B.G., Meehan, B.S., Jackson, D.C., Strugnell, R.A., Cao, H., Wang, N., Fairlie, D.P., Liu, L., Godfrey, D.I., Rossjohn, J., McCluskey, J. and Corbett, A.J. (2016) 'Mucosal-Associated Invariant T-Cell Activation and Accumulation after in Vivo Infection Depends on Microbial Riboflavin Synthesis and Co-Stimulatory Signals'. *Mucosal Immunology*, 10(1), pp. 58–68. DOI: 10.1038/mi.2016.39.

Colpitts, S.L., Dalton, N.M. and Scott, P. (2009) ‘ IL-7 Receptor Expression Provides the Potential for Long-Term Survival of Both CD62L High Central Memory T Cells and Th1 Effector Cells during Leishmania Major Infection ’. *The Journal of Immunology*, 182(9), pp. 5702–5711. DOI: 10.4049/jimmunol.0803450.

Cook, K.W., Letley, D.P., Ingram, R.J.M., Staples, E., Skjoldmose, H., Atherton, J.C. and Robinson, K. (2014) ‘CCL20/CCR6-Mediated Migration of Regulatory T Cells to the <Em>Helicobacter Pylori</Em>-Infected Human Gastric Mucosa’. *Gut*, 63(10), pp. 1550 LP – 1559. DOI: 10.1136/gutjnl-2013-306253.

Cooper, M.D., Raymond, D.A., Peterson, R.D., South, M.A. and Good, R.A. (1966) ‘The Functions of the Thymus System and the Bursa System in the Chicken.’ *The Journal of Experimental Medicine*, 123(1), pp. 75–102. DOI: 10.1084/jem.123.1.75.

Corbett, A.J., Eckle, S.B.G., Birkinshaw, R.W., Liu, L., Patel, O., Mahony, J., Chen, Z., Reantragoon, R., Meehan, B., Cao, H., Williamson, N.A., Strugnell, R.A., Van Sinderen, D., Mak, J.Y.W., Fairlie, D.P., Kjer-Nielsen, L., Rossjohn, J. and McCluskey, J. (2014) ‘T-Cell Activation by Transitory Neo-Antigens Derived from Distinct Microbial Pathways’. *Nature*, 509(7500), pp. 361–365. DOI: 10.1038/nature13160.

Cosmi, L., Liotta, F., Lazzeri, E., Francalanci, M., Angeli, R., Mazzinghi, B., Santarlasci, V., Manetti, R., Vanini, V., Romagnani, P., Maggi, E., Romagnani, S. and Annunziato, F. (2003) ‘Human CD8+CD25+ Thymocytes Share Phenotypic and Functional Features with CD4+CD25+ Regulatory Thymocytes’. *Blood*, 102(12), pp. 4107–4114. DOI: 10.1182/blood-2003-04-1320.

Couper, K.N., Blount, D.G. and Riley, E.M. (2008) ‘IL-10: The Master Regulator of Immunity to Infection’. *The Journal of Immunology*, 180(9), pp. 5771–5777. DOI: 10.4049/jimmunol.180.9.5771.

Croxford, J.L., Miyake, S., Huang, Y.Y., Shimamura, M. and Yamamura, T. (2006) 'Invariant Vα19i T Cells Regulate Autoimmune Inflammation'. *Nature Immunology*, 7(9), pp. 987–994. DOI: 10.1038/ni1370.

Cupedo, T., Nagasawa, M., Weijer, K., Blom, B. and Spits, H. (2005) 'Development and Activation of Regulatory T Cells in the Human Fetus'. *European Journal of Immunology*, 35(2), pp. 383–390. DOI: 10.1002/eji.200425763.

Curtsinger, J.M., Schmidt, C.S., Mondino, A., Lins, D.C., Kedl, R.M., Jenkins, M.K. and Mescher, M.F. (1999) 'Inflammatory Cytokines Provide a Third Signal for Activation of Naive CD4+ and CD8+ T Cells.' *Journal of Immunology (Baltimore, Md. : 1950)*, 162(6), pp. 3256–62. DOI: 10.1007/s13398-014-0173-7.2.

Dalod, M., Chelbi, R., Malissen, B. and Lawrence, T. (2014) 'Dendritic Cell Maturation: Functional Specialization through Signaling Specificity and Transcriptional Programming'. *EMBO Journal*, 33(10), pp. 1104–1116. DOI: 10.1002/embj.201488027.

Darrasse-Jèze, G., Marodon, G., Salomon, B.L., Catala, M. and Klatzmann, D. (2005) 'Ontogeny of CD4+CD25+ Regulatory/Suppressor T Cells in Human Fetuses'. *Blood*, 105(12), pp. 4715–4721. DOI: 10.1182/blood-2004-10-4051.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S. and Reis E Sousa, C. (2004) 'Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA'. *Science*, 303(5663), pp. 1529–1531. DOI: 10.1126/science.1093616.

Doran, E., Cai, F., Holweg, C.T.J., Wong, K., Brumm, J. and Arron, J.R. (2017) Available at: https://www.frontiersin.org/article/10.3389/fmed.2017.00139.

Doyle, C. and Strominger, J.L. (1987) ‘Interaction between CD4 and Class II MHC Molecules Mediates Cell Adhesion’. *Nature*, 330(6145), pp. 256–259. DOI: 10.1038/330256a0.

Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F. and Campbell, D.J. (2012) ‘Functionally Distinct Subsets of Human FOXP3 + Treg Cells That Phenotypically Mirror Effector Th Cells’. *Blood*, 119(19), pp. 4430–4440. DOI: 10.1182/blood-2011-11-392324.

Dusseaux, M., Martin, E., Serriari, N., Péguillet, I., Premel, V., Louis, D., Milder, M., Le Bourhis, L., Soudais, C., Treiner, E. and Lantz, O. (2011) ‘Human MAIT Cells Are Xenobiotic-Resistant, Tissue-Targeted, CD161 Hi IL-17-Secreting T Cells’. *Blood*, 117(4), pp. 1250–1259. DOI: 10.1182/blood-2010-08-303339.

Eckle, S.B.G., Birkinshaw, R.W., Kostenko, L., Corbett, A.J., McWilliam, H.E.G., Reantragoon, R., Chen, Z., Gherardin, N.A., Beddoe, T., Liu, L., Patel, O., Meehan, B., Fairlie, D.P., Villadangos, J.A., Godfrey, D.I., Kjer-Nielsen, L., McCluskey, J. and Rossjohn, J. (2014) ‘A Molecular Basis Underpinning the T Cell Receptor Heterogeneity of Mucosal-Associated Invariant T Cells’. *Journal of Experimental Medicine*, 211(8), pp. 1585–1600. DOI: 10.1084/jem.20140484.

Eckle, S.B.G., Corbett, A.J., Keller, A.N., Chen, Z., Godfrey, D.I., Liu, L., Mak, J.Y.W., Fairlie, D.P., Rossjohn, J. and McCluskey, J. (2015) ‘Recognition of Vitamin B Precursors and Byproducts by Mucosal Associated Invariant T Cells’. *Journal of Biological Chemistry*, 290(51), pp. 30204–30211. DOI: 10.1074/jbc.R115.685990.

Eisenbarth, S.C. (2019) ‘Dendritic Cell Subsets in T Cell Programming: Location Dictates Function’. *Nature Reviews Immunology*, 19(2), pp. 89–103. DOI: 10.1038/s41577-018-0088-1.

Fogg, D.K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D.R., Cumano, A. and Geissmann, F. (2006) 'A Clonogenic Bone Harrow Progenitor Specific for Macrophages and Dendritic Cells'. *Science*, 311(5757), pp. 83–87. DOI: 10.1126/science.1117729.

Fontenot, J.D., Rasmussen, J.P., Williams, L.M., Dooley, J.L., Farr, A.G. and Rudensky, A.Y. (2005) 'Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor Foxp3'. *Immunity*, 22(3), pp. 329–341. DOI: 10.1016/j.immuni.2005.01.016.

Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Müller, I., Wolf, E. and Lipp, M. (1999) 'CCR7 Coordinates the Primary Immune Response by Establishing Functional Microenvironments in Secondary Lymphoid Organs'. *Cell*, 99(1), pp. 23–33. DOI: 10.1016/S0092-8674(00)80059-8.

Förster, R., Davalos-Misslitz, A.C. and Rot, A. (2008) 'CCR7 and Its Ligands: Balancing Immunity and Tolerance'. *Nature Reviews Immunology*, 8(5), pp. 362–371. DOI: 10.1038/nri2297.

Fujii, S.-I., Shimizu, K., Smith, C., Bonifaz, L. and Steinman, R.M. (2003) 'Activation of Natural Killer T Cells by Alpha-Galactosylceramide Rapidly Induces the Full Maturation of Dendritic Cells in Vivo and Thereby Acts as an Adjuvant for Combined CD4 and CD8 T Cell Immunity to a Coadministered Protein.' *The Journal of Experimental Medicine*, 198(2), pp. 267–279. DOI: 10.1084/jem.20030324.

Fujii, S.-I., Liu, K., Smith, C., Bonito, A.J. and Steinman, R.M. (2004) 'The Linkage of Innate to Adaptive Immunity via Maturing Dendritic Cells in Vivo Requires CD40 Ligation in Addition to Antigen Presentation and CD80/86 Costimulation.' *The Journal of Experimental Medicine*, 199(12), pp. 1607–18. DOI: 10.1084/jem.20040317.

Fujii, S.I., Shimizu, K., Hemmi, H. and Steinman, R.M. (2007) 'Innate Vα14+ Natural Killer T Cells Mature Dendritic Cells, Leading to Strong Adaptive Immunity'. *Immunological Reviews*, 220(1), pp. 183–198. DOI: 10.1111/j.1600-065X.2007.00561.x.

Fujii, Y., Okumura, M., Inada, K., Nakahara, K. and Matsuda, H. (1992) 'CD45 Isoform Expression during T Cell Development in the Thymus'. *European Journal of Immunology*, 22(7), pp. 1843–1850. DOI: 10.1002/eji.1830220725.

Fukuhara, K., Okumura, M., Shiono, H., Inoue, M., Kadota, Y., Miyoshi, S. and Matsuda, H. (2002) 'A Study on CD45 Isoform Expression during T-Cell Development and Selection Events in the Human Thymus'. *Human Immunology*, 63(5), pp. 394–404. DOI: 10.1016/S0198-8859(02)00379-8.

Gherardin, N.A., Keller, A.N., Woolley, R.E., Le Nours, J., Ritchie, D.S., Neeson, P.J., Birkinshaw, R.W., Eckle, S.B.G., Waddington, J.N., Liu, L., Fairlie, D.P., Uldrich, A.P., Pellicci, D.G., McCluskey, J., Godfrey, D.I. and Rossjohn, J. (2016) 'Diversity of T Cells Restricted by the MHC Class I-Related Molecule MR1 Facilitates Differential Antigen Recognition'. *Immunity*, 44(1), pp. 32–45. DOI: 10.1016/j.immuni.2015.12.005.

Gherardin, N.A., Souter, M.N.T., Koay, H.F., Mangas, K.M., Seemann, T., Stinear, T.P., Eckle, S.B.G., Berzins, S.P., d'Udekem, Y., Konstantinov, I.E., Fairlie, D.P., Ritchie, D.S., Neeson, P.J., Pellicci, D.G., Uldrich, A.P., McCluskey, J. and Godfrey, D.I. (2018) 'Human Blood MAIT Cell Subsets Defined Using MR1 Tetramers'. *Immunology and Cell Biology*, 96(5), pp. 507–525. DOI: 10.1111/imcb.12021.

Godfrey, D.I., Kennedy, J., Suda, T. and Zlotnik, A. (1993) 'A Developmental Pathway Involving Four Phenotypically and Functionally Distinct Subsets of CD3-CD4-CD8- Triple-Negative Adult Mouse Thymocytes Defined by CD44 and CD25 Expression.' *Journal of Immunology (Baltimore, Md. : 1950)*, 150(10), pp. 4244–4252. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8387091.

Godfrey, D.I., Uldrich, A.P., Mccluskey, J., Rossjohn, J. and Moody, D.B. (2015) 'The Burgeoning Family of Unconventional T Cells'. *Nature Immunology*, 16(11), pp. 1114–1123. DOI: 10.1038/ni.3298.

Godfrey, D.I. and Kronenberg, M. (2004) 'Going Both Ways: Immune Regulation via CD1d-Dependent NKT Cells'. *Journal of Clinical Investigation*, 114(10), pp. 1379–1388. DOI: 10.1172/JCI200423594.

Godfrey, D.I., Koay, H.-F., McCluskey, J. and Gherardin, N.A. (2019) 'The Biology and Functional Importance of MAIT Cells'. *Nature Immunology*, 20(9), pp. 1110–1128. DOI: 10.1038/s41590-019-0444-8.

Griffith, J.W., Sokol, C.L. and Luster, A.D. (2014) 'Chemokines and Chemokine Receptors: Positioning Cells for Host Defense and Immunity'. *Annual Review of Immunology*, 32(1), pp. 659–702. DOI: 10.1146/annurev-immunol-032713-120145.

Groom, J.R. and Luster, A.D. (2011) 'CXCR3 in T Cell Function'. *Experimental Cell Research*, 317(5), pp. 620–631. DOI: 10.1016/j.yexcr.2010.12.017.

Guo, X., Tanaka, Y. and Kondo, M. (2015) 'Thymic Precursors of TCRαβ+CD8αα+ Intraepithelial Lymphocytes Are Negative for CD103'. *Immunology Letters*, 163(1), pp. 40–48. DOI: 10.1016/j.imlet.2014.11.007.

Den Haan, J.M.M., Lehar, S.M. and Bevan, M.J. (2000a) ‘CD8+ but Not CD8- Dendritic Cells Cross-Prime Cytotoxic T Cells in Vivo’. *Journal of Experimental Medicine*, 192(12), pp. 1685–1695. DOI: 10.1084/jem.192.12.1685.

Den Haan, J.M.M., Lehar, S.M. and Bevan, M.J. (2000b) ‘CD8+ but Not CD8- Dendritic Cells Cross-Prime Cytotoxic T Cells in Vivo’. *Journal of Experimental Medicine*. DOI: 10.1084/jem.192.12.1685.

Häcker, H., Mischak, H., Miethke, T., Liptay, S., Schmid, R., Sparwasser, T., Heeg, K., Lipford, G.B. and Wagner, H. (1998) ‘CpG-DNA-Specific Activation of Antigen-Presenting Cells Requires Stress Kinase Activity and Is Preceded by Non-Specific Endocytosis and Endosomal Maturation’. *EMBO Journal*, 17(21), pp. 6230–6240. DOI: 10.1093/emboj/17.21.6230.

Hamilton-Williams, E.E., Lang, A., Benke, D., Davey, G.M., Wiesmüller, K.-H. and Kurts, C. (2005) ‘Cutting Edge: TLR Ligands Are Not Sufficient to Break Cross-Tolerance to Self-Antigens’. *The Journal of Immunology*, 174(3), pp. 1159–1163. DOI: 10.4049/jimmunol.174.3.1159.

Harriff, M.J., McMurtrey, C., Froyd, C.A., Jin, H., Cansler, M., Null, M., Worley, A., Meermeier, E.W., Swarbrick, G., Nilsen, A., Lewinsohn, D.A., Hildebrand, W., Adams, E.J. and Lewinsohn, D.M. (2018) ‘MR1 Displays the Microbial Metabolome Driving Selective MR1-Restricted T Cell Receptor Usage’. *Science Immunology*, 3(25). DOI: 10.1126/sciimmunol.aao2556.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M. and Aderem, A. (2001) ‘The Innate Immune Response to Bacterial Flagellin Is Mediated by Toll-like Receptor 5’. *Nature*, 410(6832), pp. 1099–1103. DOI: 10.1038/35074106.

Held, K., Beltrán, E., Moser, M., Hohlfeld, R. and Dornmair, K. (2015) 'T-Cell Receptor Repertoire of Human Peripheral CD161hiTRAV1-2+ MAIT Cells Revealed by next Generation Sequencing and Single Cell Analysis'. *Human Immunology*, 76(9), pp. 607–614. DOI: 10.1016/j.humimm.2015.09.002.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000) 'A Toll-like Receptor Recognizes Bacterial DNA'. *Nature*, 408(6813), pp. 740–745. DOI: 10.1038/35047123.

Hermans, Ian F., Silk, J.D., Gileadi, U., Salio, M., Mathew, B., Ritter, G., Schmidt, R., Harris, A.L., Old, L. and Cerundolo, V. (2003) ' NKT Cells Enhance CD4 + and CD8 + T Cell Responses to Soluble Antigen In Vivo through Direct Interaction with Dendritic Cells '. *The Journal of Immunology*, 171(10), pp. 5140–5147. DOI: 10.4049/jimmunol.171.10.5140.

Hettinger, J., Richards, D.M., Hansson, J., Barra, M.M., Joschko, A.C., Krijgsveld, J. and Feuerer, M. (2013) 'Origin of Monocytes and Macrophages in a Committed Progenitor'. *Nature Immunology*, 14(8), pp. 821–830. DOI: 10.1038/ni.2638.

Hildner, K., Edelson, B.T., Purtha, W.E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B.U., Unanue, E.R., Diamond, M.S., Schreiber, R.D., Murphy, T.L. and Murphy, K.M. (2008) 'Batf3 Deficiency Reveals a Critical Role for CD8α + Dendritic Cells in Cytotoxic T Cell Immunity'. *Science*, 322(5904), pp. 1097–1100. DOI: 10.1126/science.1164206.

Himmel, M.E., Crome, S.Q., Ivison, S., Piccirillo, C., Steiner, T.S. and Levings, M.K. (2011) 'Human CD4+FOXP3+ Regulatory T Cells Produce CXCL8 and Recruit Neutrophils'. *European Journal of Immunology*, 41(2), pp. 306–312. DOI: 10.1002/eji.201040459.

Hinks, T.S.C., Zhou, X., Staples, K.J., Dimitrov, B.D., Manta, A., Petrossian, T., Lum, P.Y., Smith, C.G., Ward, J.A., Howarth, P.H., Walls, A.F., Gadola, S.D. and Djukanović, R. (2015) 'Innate and Adaptive T Cells in Asthmatic Patients: Relationship to Severity and Disease Mechanisms'. *Journal of Allergy and Clinical Immunology*, 136(2), pp. 323–333. DOI: https://doi.org/10.1016/j.jaci.2015.01.014.

Holdsworth, S.R. and Can, P.Y. (2015) 'Cytokines: Names and Numbers You Should Care About'. *Clinical Journal of the American Society of Nephrology*, 10(12), pp. 2243–2254. DOI: 10.2215/CJN.07590714.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999) 'Cutting Edge: Toll-Like Receptor 4 (TLR4)-Deficient Mice Are Hyporesponsive to Lipopolysaccharide: Evidence for TLR4 as the <Em>Lps</Em> Gene Product'. *The Journal of Immunology*, 162(7), pp. 3749 LP – 3752. Available at: http://www.jimmunol.org/content/162/7/3749.abstract.

Hsieh, C.S., Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'Garra, A. and Murphy, K.M. (1993) 'Development of T H 1 CD4 + T Cells through IL-12 Produced by Listeria-Induced Macrophages'. *Science*, 260(5107), pp. 547–549. DOI: 10.1126/science.8097338.

Huang, S., Gilfillan, S., Kim, S., Thompson, B., Wang, X., Sant, A.J., Fremont, D.H., Lantz, O. and Hansen, T.H. (2008) 'MR1 Uses an Endocytic Pathway to Activate Mucosal-Associated Invariant T Cells'. *Journal of Experimental Medicine*, 205(5), pp. 1201–1211. DOI: 10.1084/jem.20072579.

Husmann, L.A. and Bevan, M.J. (1988) 'Cooperation between Helper T Cells and Cytotoxic T Lymphocyte Precursors'. *Annals of the New York Academy of Sciences*, 532(1), pp. 158–169. DOI: 10.1111/j.1749-6632.1988.tb36335.x.

Iellem, A., Mariani, M., Lang, R., Recalde, H., Panina-Bordignon, P., Sinigaglia, F. and D'Ambrosio, D. (2001) 'Unique Chemotactic Response Profile and Specific Expression of Chemokine Receptors CCR4 and CCR8 by CD4+CD25+ Regulatory T Cells'. *Journal of Experimental Medicine*, 194(6), pp. 847–853. DOI: 10.1084/jem.194.6.847.

Imai, T., Nagira, M., Takagi, S., Kakizaki, M., Nishimura, M., Wang, J., Gray, P.W., Matsushima, K. and Yoshie, O. (1999) 'Selective Recruitment of CCR4-Bearing T(h)2 Cells toward Antigen-Presenting Cells by the CC Chemokines Thymus and Activation-Regulated Chemokine and Macrophage-Derived Chemokine'. *International Immunology*, 11(1), pp. 81–88. DOI: 10.1093/intimm/11.1.81.

Ito, T., Hanabuchi, S., Wang, Y.H., Park, W.R., Arima, K., Bover, L., Qin, F.X.F., Gilliet, M. and Liu, Y.J. (2008) 'Two Functional Subsets of FOXP3 + Regulatory T Cells in Human Thymus and Periphery'. *Immunity*, 28(6), pp. 870–880. DOI: 10.1016/j.immuni.2008.03.018.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J. and Littman, D.R. (2006) 'The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells'. *Cell*, 126(6), pp. 1121–1133. DOI: 10.1016/j.cell.2006.07.035.

Janssen, E.M., Droin, N.M., Lemmens, E.E., Pinkoski, M.J., Bensinger, S.J., Ehst, B.D., Griffith, T.S., Green, D.R. and Schoenberger, S.P. (2005) 'CD4+ T-Cell Help Controls CD8+ T-Cell Memory via TRAIL-Mediated Activation-Induced Cell Death'. *Nature*, 434(7029), pp. 88–93. DOI: 10.1038/nature03337.

Kaech, S.M., Wherry, E.J. and Ahmed, R. (2002) 'Effector and Memory T-Cell Differentiation: Implications for Vaccine Development'. *Nature Reviews Immunology*, 2(4), pp. 251–262. DOI: 10.1038/nri778.

Kara, E.E., Comerford, I., Fenix, K.A., Bastow, C.R., Gregor, C.E., McKenzie, D.R. and McColl, S.R. (2014) 'Tailored Immune Responses: Novel Effector Helper T Cell Subsets in Protective Immunity'. *PLoS Pathogens*, 10(2). DOI: 10.1371/journal.ppat.1003905.

Keir, M.E., Freeman, G.J. and Sharpe, A.H. (2007) 'PD-1 Regulates Self-Reactive CD8+ T Cell Responses to Antigen in Lymph Nodes and Tissues'. *The Journal of Immunology*, 179(8), pp. 5064–5070. DOI: 10.4049/jimmunol.179.8.5064.

Kelly, J., Minoda, Y., Meredith, T., Cameron, G., Philipp, M., Pellicci, D.G., Corbett, A.J., Kurts, C., Gray, D.H., Godfrey, D.I., Kannourakis, G. and Berzins, S.P. (2019) 'Chronically Stimulated Human MAIT Cells Are Unexpectedly Potent IL-13 Producers'. *Immunology & Cell Biology*. DOI: 10.1111/imcb.12281.

Kjer-Nielsen, L. *et al.* (2012) 'MR1 Presents Microbial Vitamin B Metabolites to MAIT Cells'. *Nature*, 14(12), p. 773. DOI: 10.1038/nature11605.

Knox, J.J., Cosma, G.L., Betts, M.R. and McLane, L.M. (2014) 'Characterization of T-Bet and Eomes in Peripheral Human Immune Cells'. *Frontiers in Immunology*, 5(217). DOI: 10.3389/fimmu.2014.00217.

Koay, H.F. *et al.* (2016) 'A Three-Stage Intrathymic Development Pathway for the Mucosal-Associated Invariant T Cell Lineage'. *Nature Immunology*, 17(11), pp. 1300–1311. DOI: 10.1038/ni.3565.

Koay, H.F., Gherardin, N.A., Xu, C., Seneviratna, R., Zhao, Z., Chen, Z., Fairlie, D.P., McCluskey, J., Pellicci, D.G., Uldrich, A.P. and Godfrey, D.I. (2019) 'Diverse MR1-Restricted T Cells in Mice and Humans'. *Nature Communications*, 10(2243). DOI: 10.1038/s41467-019-10198-w.

Koch, M.A., Tucker-Heard, G., Perdue, N.R., Killebrew, J.R., Urdahl, K.B. and Campbell, D.J. (2009) 'The Transcription Factor T-Bet Controls Regulatory T Cell Homeostasis and Function during Type 1 Inflammation'. *Nature Immunology*, 10(6), pp. 595–602. DOI: 10.1038/ni.1731.

Koch, U. and Radtke, F. (2011) 'Mechanisms of T Cell Development and Transformation'. *Annual Review of Cell and Developmental Biology*, 27(1), pp. 539–562. DOI: 10.1146/annurev-cellbio-092910-154008.

Kondo, M., Weissman, I.L. and Akashi, K. (1997) 'Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow'. *Cell*, 91(5), pp. 661–672. DOI: 10.1016/S0092-8674(00)80453-5.

Kovalovsky, D., Uche, O.U., Eladad, S., Hobbs, R.M., Yi, W., Alonzo, E., Chua, K., Eidson, M., Kim, H.J., Im, J.S., Pandolfi, P.P. and Sant'Angelo, D.B. (2008) 'The BTB-Zinc Finger Transcriptional Regulator PLZF Controls the Development of Invariant Natural Killer T Cell Effector Functions'. *Nature Immunology*, 9(9), pp. 1055–1064. DOI: 10.1038/ni.1641.

Krebs, C.F. *et al.* (2016) 'Autoimmune Renal Disease Is Exacerbated by S1P-Receptor-1-Dependent Intestinal Th17 Cell Migration to the Kidney'. *Immunity*, 45(5), pp. 1078–1092. DOI: 10.1016/j.immuni.2016.10.020.

Krishnaswamy, J.K. *et al.* (2017) 'Migratory CD11b+ Conventional Dendritic Cells Induce T Follicular Helper Cell–Dependent Antibody Responses'. *Science Immunology*, 2(18). DOI: 10.1126/sciimmunol.aam9169.

Kunkel, E.J., Boisvert, J., Murphy, K., Vierra, M.A., Genovese, M.C., Wardlaw, A.J., Greenberg, H.B., Hodge, M.R., Wu, L., Butcher, E.C. and Campbell, J.J. (2002) 'Expression of the Chemokine Receptors CCR4, CCR5, and CXCR3 by Human Tissue-Infiltrating Lymphocytes'. *The American Journal of Pathology*, 160(1), pp. 347–355. DOI: 10.1016/S0002-9440(10)64378-7.

Kurioka, A., Ussher, J.E., Cosgrove, C., Clough, C., Fergusson, J.R., Smith, K., Kang, Y.H., Walker, L.J., Hansen, T.H., Willberg, C.B. and Klenerman, P. (2015) 'MAIT Cells Are Licensed through Granzyme Exchange to Kill Bacterially Sensitized Targets'. *Mucosal Immunology*, 8(2), pp. 429–440. DOI: 10.1038/mi.2014.81.

Kurts, C., Heath, W.R., Carbone, F.R., Allison, J., Miller, J.F.A.F., Kosaka, H. and Victoria. (1996) 'Constitutive Class I-Restricted Exogenous Presentation of Self Antigens in Vivo'. *Journal of Experimental Medicine*, 184(3), pp. 923–930. DOI: 10.1084/jem.184.3.923.

Kurts, C., Robinson, B.W.S. and Knolle, P. a. (2010) 'Cross-Priming in Health and Disease.' *Nature Reviews. Immunology*, 10(6), pp. 403–14. DOI: 10.1038/nri2780.

Lepore, M., Kalinichenko, A., Calogero, S., Kumar, P., Paleja, B., Schmaler, M., Narang, V., Zolezzi, F., Poidinger, M., Mori, L. and de Libero, G. (2017) 'Functionally Diverse Human T Cells Recognize Non-Microbial Antigens Presented by MR1'. *ELife*, 6. DOI: 10.7554/eLife.24476.

Liu, K. and Nussenzweig, M.C. (2010) 'Origin and Development of Dendritic Cells'. *Immunological Reviews*, 234(1), pp. 45–54. DOI: 10.1111/j.0105-2896.2009.00879.x.

Lord, G.M., Rao, R.M., Choe, H., Sullivan, B.M., Lichtman, A.H., Luscinskas, F.W. and Glimcher, L.H. (2005) 'T-Bet Is Required for Optimal Proinflammatory CD4+ T-Cell Trafficking'. *Blood*, 106(10), pp. 3432–3439. DOI: 10.1182/blood-2005-04-1393.

Lu, Y.C., Yeh, W.C. and Ohashi, P.S. (2008) 'LPS/TLR4 Signal Transduction Pathway'. *Cytokine*, 42(2), pp. 145–151. DOI: 10.1016/j.cyto.2008.01.006.

Luckheeram, R.V., Zhou, R., Verma, A.D. and Xia, B. (2012) 'CD4 +T Cells: Differentiation and Functions'. *Clinical and Developmental Immunology*, 2012(925135). DOI: 10.1155/2012/925135.

Maddur, M.S., Miossec, P., Kaveri, S. V. and Bayry, J. (2012) 'Th17 Cells: Biology, Pathogenesis of Autoimmune and Inflammatory Diseases, and Therapeutic Strategies'. *American Journal of Pathology*, 181(1), pp. 8–18. DOI: 10.1016/j.ajpath.2012.03.044.

Martin, E., Treiner, E., Duban, L., Guerri, L., Laude, H., Toly, C., Premel, V., Devys, A., Moura, I.C., Tilloy, F., Cherif, S., Vera, G., Latour, S., Soudais, C. and Lantz, O. (2009) 'Stepwise Development of Mait Cells in Mouse and Human'. *PLoS Biology*, 7(3), pp. 0525–0536. DOI: 10.1371/journal. pbio.1000054.

Matsuoka, K., Inoue, N., Sato, T., Okamoto, S., Hisamatsu, T., Kishi, Y., Sakuraba, A., Hitotsumatsu, O., Ogata, H., Koganei, K., Fukushima, T., Kanai, T., Watanabe, M., Ishii, H. and Hibi, T. (2004) 'T-Bet Upregulation and Subsequent Interleukin 12 Stimulation Are Essential for Induction or Th1 Mediated Immunopathology in Crohn's Disease'. *Gut*, 53(9), pp. 1303–1308. DOI: 10.1136/gut.2003.024190.

Maurer, T., Heit, A., Hochrein, H., Ampenberger, F., O'Keeffe, M., Bauer, S., Lipford, G.B., Vabulas, R.M. and Wagner, H. (2002) 'CpG-DNA Aided Cross-Presentation of Soluble Antigens by Dendritic Cells'. *European Journal of Immunology*, 32(8), pp. 2356–2364. DOI: 10.1002/1521-4141(200208)32:8<2356::AID-IMMU2356>3.0.CO;2-Z.

McGettrick, A.F. and O'Neill, L.A. (2010) 'Localisation and Trafficking of Toll-like Receptors: An Important Mode of Regulation'. *Current Opinion in Immunology*, 22(1), pp. 20–27. DOI: 10.1016/j.coi.2009.12.002.

McWilliam, H.E.G., Eckle, S.B.G., Theodossis, A., Liu, L., Chen, Z., Wubben, J.M., Fairlie, D.P., Strugnell, R.A., Mintern, J.D., McCluskey, J., Rossjohn, J. and Villadangos, J.A. (2016) 'The Intracellular Pathway for the Presentation of Vitamin B-Related Antigens by the Antigen-Presenting Molecule MR1'. *Nature Immunology*, 17(5), pp. 531–537. DOI: 10.1038/ni.3416.

Meermeier, E.W., Laugel, B.F., Sewell, A.K., Corbett, A.J., Rossjohn, J., McCluskey, J., Harriff, M.J., Franks, T., Gold, M.C. and Lewinsohn, D.M. (2016) 'Human TRAV1-2-Negative MR1-Restricted T Cells Detect S. Pyogenes and Alternatives to MAIT Riboflavin-Based Antigens'. *Nature Communications*, 7(12506). DOI: 10.1038/ncomms12506.

Meierovics, A.I. and Cowley, S.C. (2016) 'MAIT Cells Promote Inflammatory Monocyte Differentiation into Dendritic Cells during Pulmonary Intracellular Infection'. *Journal of Experimental Medicine*, pp. 2793–2809. DOI: 10.1084/jem.20160637.

Michelsen, K.S., Aicher, A., Mohaupt, M., Hartung, T., Dimmeler, S., Kirschning, C.J. and Schumann, R.R. (2001) 'The Role of Toll-like Receptors (TLRs) in Bacteria-Induced Maturation of Murine Dendritic Cells (DCs): Peptidoglycan and Lipoteichoic Acid Are Inducers of DC Maturation and Require TLR2'. *Journal of Biological Chemistry*, 276(28), pp. 25680–25686. DOI: 10.1074/jbc.M011615200.

Michie, C.A., McLean, A., Alcock, C. and Beverley, P.C.L. (1992) 'Lifespan of Human Lymphocyte Subsets Defined by CD45 Isoforms'. *Nature*, 360(6401), pp. 264–265. DOI: 10.1038/360264a0.

Miller, J.F.A.P. (1961) ‘Immunological Function of the Thymus’. *The Lancet*, 278(7205), pp. 748–749. DOI: 10.1016/S0140-6736(61)90693-6.

Miyazaki, Y., Miyake, S., Chiba, A., Lantz, O. and Yamamura, T. (2011) ‘Mucosal-Associated Invariant T Cells Regulate Th1 Response in Multiple Sclerosis’. *International Immunology*, 23(9), pp. 529–535. DOI: 10.1093/intimm/dxr047.

Moore, M.W., Carbone, F.R. and Bevan, M.J. (1988) ‘Introduction of Soluble Protein into the Class I Pathway of Antigen Processing and Presentation’. *Cell*, 54(6), pp. 777–785. DOI: 10.1016/S0092-8674(88)91043-4.

Murphy, K.M., Travers, P. and Walport, M. (2012) *Janeway’s Immunology*.

Naik, S.H., Sathe, P., Park, H.Y., Metcalf, D., Proietto, A.I., Dakic, A., Carotta, S., O’Keeffe, M., Bahlo, M., Papenfuss, A., Kwak, J.Y., Wu, L. and Shortman, K. (2007) ‘Development of Plasmacytoid and Conventional Dendritic Cell Subtypes from Single Precursor Cells Derived in Vitro and in Vivo’. *Nature Immunology*, 8(11), pp. 1217–1226. DOI: 10.1038/ni1522.

Nakano, H., Burgents, J.E., Nakano, K., Whitehead, G.S., Cheong, C., Bortner, C.D. and Cook, D.N. (2013) ‘Migratory Properties of Pulmonary Dendritic Cells Are Determined by Their Developmental Lineage.’ *Mucosal Immunology*, 6(4), pp. 678–91. DOI: 10.1038/mi.2012.106.

Nishimura, T., Kitamura, H., Iwakabe, K., Yahata, T., Ohta, a., Sato, M., Takeda, K., Okumura, K., Van Kaer, L., Kawano, T., Taniguchi, M., Nakui, M., Sekimoto, M. and Koda, T. (2000) ‘The Interface between Innate and Acquired Immunity: Glycolipid Antigen Presentation by CD1d-Expressing Dendritic Cells to NKT Cells Induces the Differentiation of Antigen-Specific Cytotoxic T Lymphocytes.’ *International Immunology*, 12(7), pp. 987–994. DOI: 10.1093/intimm/12.7.987.

Norment, A.M., Salter, R.D., Parham, P., Engelhard, V.H. and Littman, D.R. (1988) 'Cell-Cell Adhesion Mediated by CD8 and MHC Class I Molecules'. *Nature*, 336(6194), pp. 79–81. DOI: 10.1038/336079a0.

Nunes-Cabaço, H., Caramalho, Í., Sepúlveda, N. and Sousa, A.E. (2011) 'Differentiation of Human Thymic Regulatory T Cells at the Double Positive Stage'. *European Journal of Immunology*, 41(12), pp. 3604–3614. DOI: 10.1002/eji.201141614.

Nussenzweig, M.C., Steinman, R.M., Gutchinov, B. and Cohn, Z.A. (1980) 'Dendritic Cells Are Accessory Cells for the Development of Anti-Trinitrophenyl Cytotoxic T Lymphocytes.' *The Journal of Experimental Medicine*, 152(4), pp. 1070–1084. DOI: 10.1084/jem.152.4.1070.

van Oers, N.S. (1995) 'The Pre-T Cell Receptor (TCR) Complex Is Functionally Coupled to the TCR-Zeta Subunit'. *Journal of Experimental Medicine*. DOI: 10.1084/jem.182.5.1585.

Van Oers, N.S.C., Lowin-Kropf, B., Finlay, D., Connolly, K. and Weiss, A. (1996) 'Aβ T Cell Development Is Abolished in Mice Lacking Both Lck and Fyn Protein Tyrosine Kinases'. *Immunity*, 5(5), pp. 429–436. DOI: 10.1016/S1074-7613(00)80499-9.

Ouyang, W., Rutz, S., Crellin, N.K., Valdez, P.A. and Hymowitz, S.G. (2011) 'Regulation and Functions of the IL-10 Family of Cytokines in Inflammation and Disease'. *Annual Review of Immunology*, 29(1), pp. 71–109. DOI: 10.1146/annurev-immunol-031210-101312.

Pennock, N.D., White, J.T., Cross, E.W., Cheney, E.E., Tamburini, B.A. and Kedl, R.M. (2013) 'T Cell Responses: Naïve to Memory and Everything in Between'. *American Journal of Physiology - Advances in Physiology Education*, 37(4), pp. 273–283. DOI: 10.1152/advan.00066.2013.

Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., Vanhoutte, L., Neyt, K., Killeen, N., Malissen, B., Hammad, H. and Lambrecht, B.N. (2013) 'Conventional and Monocyte-Derived CD11b+ Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen'. *Immunity*, 38(2), pp. 322–335. DOI: 10.1016/j.immuni.2012.10.016.

Pooley, J.L., Heath, W.R. and Shortman, K. (2001) ' Cutting Edge: Intravenous Soluble Antigen Is Presented to CD4 T Cells by CD8 − Dendritic Cells, but Cross-Presented to CD8 T Cells by CD8 + Dendritic Cells '. *The Journal of Immunology*, 166(9), pp. 5327–5330. DOI: 10.4049/jimmunol.166.9.5327.

Porcelli, S., Yockey, C.E., Brenner, M.B. and Balk, S.P. (1993) 'Analysis of T Cell Antigen Receptor (TCR) Expression by Human Peripheral Blood CD4-8- Alpha/Beta T Cells Demonstrates Preferential Use of Several V Beta Genes and an Invariant TCR Alpha Chain.' *The Journal of Experimental Medicine*, 178(1), pp. 1–16. DOI: 10.1084/jem.178.1.1.

Rahimpour, A., Koay, H.F., Enders, A., Clanchy, R., Eckle, S.B.G., Meehan, B., Chen, Z., Whittle, B., Liu, L., Fairlie, D.P., Goodnow, C.C., McCluskey, J., Rossjohn, J., Uldrich, A.P., Pellicci, D.G. and Godfrey, D.I. (2015) 'Identification of Phenotypically and Functionally Heterogeneous Mouse Mucosal-Associated Invariant T Cells Using MR1 Tetramers'. *Journal of Experimental Medicine*, 212(7), pp. 1095–1108. DOI: 10.1084/jem.20142110.

Rawal, S., Park, H.J., Chu, F., Zhang, M., Nattamai, D., Kannan, S.C., Sharma, R., Delgado, D.A., Chou, T., Davis, R.E. and Neelapu, S.S. (2011) 'Role of IL-4 in Inducing Immunosuppressive Tumor Microenvironment in Follicular Lymphoma'. *Blood*, 118(21), pp. 771 LP – 771. Available at: http://www.bloodjournal.org/content/118/21/771.abstract.

Reantragoon, R. *et al.* (2013) ‘Antigen-Loaded MR1 Tetramers Define T Cell Receptor Heterogeneity in Mucosal-Associated Invariant T Cells’. *Journal of Experimental Medicine*, 210(11), pp. 2305–2320. DOI: 10.1084/jem.20130958.

Reis, B.S., Hoytema van Konijnenburg, D.P., Grivennikov, S.I. and Mucida, D. (2014) ‘Transcription Factor T-Bet Regulates Intraepithelial Lymphocyte Functional Maturation’. *Immunity*, 41(2), pp. 244–256. DOI: 10.1016/j.immuni.2014.06.017.

Rescigno, M., Granucci, F., Citterio, S., Foti, M. and Ricciardi-Castagnoli, P. (1999) ‘Coordinated Events during Bacteria-Induced DC-Maturation’. *Immunology Today*, 20(5), pp. 200–203. DOI: 10.1016/S0167-5699(98)01427-3.

Riegert, P., Wanner, V. and Bahram, S. (1998) ‘Genomics, Isoforms, Expression, and Phylogeny of the MHC Class I-Related MR1 Gene.’ *Journal of Immunology (Baltimore, Md.: 1950)*, 161(8), pp. 4066–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9780177.

del Rio, M.-L., Rodriguez-Barbosa, J.-I., Kremmer, E. and Förster, R. (2007) ‘ CD103 − and CD103 + Bronchial Lymph Node Dendritic Cells Are Specialized in Presenting and Cross-Presenting Innocuous Antigen to CD4 + and CD8 + T Cells ’. *The Journal of Immunology*, 178(11), pp. 6861–6866. DOI: 10.4049/jimmunol.178.11.6861.

Rodríguez-Perea, A.L., Arcia, E.D., Rueda, C.M. and Velilla, P.A. (2016) ‘Phenotypical Characterization of Regulatory T Cells in Humans and Rodents’. *Clinical and Experimental Immunology*, 185(3), pp. 281–291. DOI: 10.1111/cei.12804.

Rogge, L., Barberis-Maino, L., Biffi, M., Passini, N., Presky, D.H., Gubler, U. and Sinigaglia, F. (1997) 'Selective Expression of an Interleukin-12 Receptor Component by Human T Helper 1 Cells'. *Journal of Experimental Medicine*, 185(5), pp. 825–831. DOI: 10.1084/jem.185.5.825.

Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H.J. and Von Boehmer, H. (1994) 'Analysis and Expression of a Cloned Pre-T Cell Receptor Gene'. *Science*, 266(5188), pp. 1208–1212. DOI: 10.1126/science.7973703.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M. (1995) 'Immunologic Self-Tolerance Maintained by Activated T Cells Expressing IL-2 Receptor Alpha-Chains (CD25). Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune Diseases.' *Journal of Immunology (Baltimore, Md.: 1950)*, 155(3), pp. 1151–1164. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7636184.

Salio, M., Gasser, O., Gonzalez-lopez, C., Martens, A., Veerapen, N., Gileadi, U., Jacob, G., Napolitani, G., Anderson, R., Painter, G., Besra, G.S., Hermans, I.F. and Cerundolo, V. (2017) 'Activation of Human Mucosal-Associated Invariant T Cells Induces CD40L-Dependent Maturation of Monocyte-Derived and Primary Dendritic Cells'. *Journal of Immunology (Baltimore, Md.: 1950)*. DOI: 10.4049/jimmunol.1700615.

Sallusto, F., Palermo, B., Lenig, D., Miettinen, M., Matikainen, S., Julkunen, I., Forster, R., Burgstahler, R., Lipp, M. and Lanzavecchia, A. (1999) 'Distinct Patterns and Kinetics of Chemokine Production Regulate Dendritic Cell Function'. *European Journal of Immunology*, 29(5), pp. 1617–1625. DOI: 10.1002/(SICI)1521-4141(199905)29:05<1617::AID-IMMU1617>3.0.CO;2-3.

Sallusto, F., Lenig, D., Mackay, C.R. and Lanzavecchia, A. (1998) 'Flexible Programs of Chemokine Receptor Expression on Human Polarized T Helper 1 and 2 Lymphocytes'. *Journal of Experimental Medicine*, 187(6), pp. 875–883. DOI: 10.1084/jem.187.6.875.

Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C.R., Qin, S. and Lanzavecchia, A. (1998) 'Rapid and Coordinated Switch in Chemokine Receptor Expression during Dendritic Cell Maturation'. *European Journal of Immunology*, 28(9), pp. 2760–2769. DOI: 10.1002/(SICI)1521-4141(199809)28:09<2760::AID-IMMU2760>3.0.CO;2-N.

Samelson, L.E. (2002) 'Signal Transduction Mediated by the T Cell Antigen Receptor: The Role of Adapter Proteins'. *Annual Review of Immunology*, 20, pp. 371–394. DOI: 10.1146/annurev.immunol.20.092601.111357.

Savage, A.K., Constantinides, M.G., Han, J., Picard, D., Martin, E., Li, B., Lantz, O. and Bendelac, A. (2008) 'The Transcription Factor PLZF Directs the Effector Program of the NKT Cell Lineage'. *Immunity*, 29(3), pp. 391–403. DOI: 10.1016/j.immuni.2008.07.011.

Savina, A. and Amigorena, S. (2007) 'Phagocytosis and Antigen Presentation in Dendritic Cells'. *Immunological Reviews*, 219(1), pp. 143–156. DOI: 10.1111/j.1600-065X.2007.00552.x.

Schmidt, A., Oberle, N. and Krammer, P.H. (2012) 'Molecular Mechanisms Oftreg-Mediatedt Cell Suppression'. *Frontiers in Immunology*, 3(51). DOI: 10.3389/fimmu.2012.00051.

Schoenberger, S.P., Toes, R.E.M., van der Voort, E.I.H., Offringa, R. and Melief, C.J.M. (1998) 'T-Cell Help for Cytotoxic T Lymphocytes Is Mediated by CD40–CD40L Interactions'. *Nature*. DOI: 10.1038/31002.

Schulz, O., Edwards, a D., Schito, M., Aliberti, J., Manickasingham, S., Sher, a. and Reis e Sousa, C. (2000) 'CD40 Triggering of Heterodimeric IL-12 P70 Production by Dendritic Cells in Vivo Requires a Microbial Priming Signal.' *Immunity*, 13(4), pp. 453–62. DOI: 10.1016/S1074-7613(00)00045-5.

Seach, N., Guerri, L., Le Bourhis, L., Mburu, Y., Cui, Y., Bessoles, S., Soudais, C. and Lantz, O. (2013) 'Double Positive Thymocytes Select Mucosal-Associated Invariant T Cells'. *The Journal of Immunology*, 191(12), pp. 6002–6009. DOI: 10.4049/jimmunol.1301212.

Semmling, V., Lukacs-Kornek, V., Thaiss, C.A., Quast, T., Hochheiser, K., Panzer, U., Rossjohn, J., Perlmutter, P., Cao, J., Godfrey, D.I., Savage, P.B., Knolle, P.A., Kolanus, W., Förster, I. and Kurts, C. (2010) 'Alternative Cross-Priming through CCL17-CCR4-Mediated Attraction of CTLs toward NKT Cell–Licensed DCs'. *Nature Immunology*, 11(4), pp. 313–321. DOI: 10.1038/ni.1848.

Soudais, C., Samassa, F., Sarkis, M., Le Bourhis, L., Bessoles, S., Blanot, D., Hervé, M., Schmidt, F., Mengin-Lecreulx, D. and Lantz, O. (2015) 'In Vitro and In Vivo Analysis of the Gram-Negative Bacteria–Derived Riboflavin Precursor Derivatives Activating Mouse MAIT Cells'. *The Journal of Immunology*, 194(10), pp. 4641–4649. DOI: 10.4049/jimmunol.1403224.

Sparwasser, T., Koch, E.S., Vabulas, R.M., Heeg, K., Lipford, G.B., Ellwart, J.W. and Wagner, H. (1998) 'Bacterial DNA and Immunostimulatory CpG Oligonucleotides Trigger Maturation and Activation of Murine Dendritic Cells'. *European Journal of Immunology*, 28(6), pp. 2045–2054. DOI: 10.1002/(SICI)1521-4141(199806)28:06<2045::AID-IMMU2045>3.0.CO;2-8.

Steinman, R.M., Adams, J.C. and Cohn, Z.A. (1975) 'Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice. IV. Identification and Distribution in Mouse Spleen'. *Journal of Experimental Medicine*, 141(4), pp. 804–820.

Stober, D., Jomantaitė, I., Schirmbeck, R. and Reimann, J. (2003) ‘ NKT Cells Provide Help for Dendritic Cell-Dependent Priming of MHC Class I-Restricted CD8 + T Cells In Vivo ’. *The Journal of Immunology*, 170(5), pp. 2540–2548. DOI: 10.4049/jimmunol.170.5.2540.

Sun, Z., Unutmaz, D., Zou, Y.R., Sunshine, M.J., Pierani, A., Brenner-Morton, S., Mebius, R.E. and Littman, D.R. (2000) ‘Requirement for RORγ in Thymocyte Survival and Lymphoid Organ Development’. *Science*, 288(5475), pp. 2369–2373. DOI: 10.1126/science.288.5475.2369.

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G. and Glimcher, L.H. (2000) ‘A Novel Transcription Factor, T-Bet, Directs Th1 Lineage Commitment’. *Cell*, 100(6), pp. 655–669. DOI: 10.1016/S0092-8674(00)80702-3.

Takaba, H. and Takayanagi, H. (2017) ‘The Mechanisms of T Cell Selection in the Thymus’. *Trends in Immunology*, 38(11), pp. 805–816. DOI: 10.1016/j.it.2017.07.010.

Takahama, Y. (2006) ‘Journey through the Thymus: Stromal Guides for T-Cell Development and Selection’. *Nature Reviews Immunology*, 6(2), pp. 127–135. DOI: 10.1038/nri1781.

Tamura, T., Tailor, P., Yamaoka, K., Kong, H.J., Tsujimura, H., O’Shea, J.J., Singh, H. and Ozato, K. (2005) ‘IFN Regulatory Factor-4 and -8 Govern Dendritic Cell Subset Development and Their Functional Diversity’. *The Journal of Immunology*, 174(5), pp. 2573–2581. DOI: 10.4049/jimmunol.174.5.2573.

Terabe, M., Park, J.M. and Berzofsky, J.A. (2004) ‘Role of IL-13 in Regulation of Anti-Tumor Immunity and Tumor Growth’. *Cancer Immunology, Immunotherapy*, 53(2), pp. 79–85. DOI: 10.1007/s00262-003-0445-0.

Thiault, N., Darrigues, J., Adoue, V., Gros, M., Binet, B., Perals, C., Leobon, B., Fazilleau, N., Joffre, O.P., Robey, E.A., Van Meerwijk, J.P.M. and Romagnoli, P. (2015) 'Peripheral Regulatory T Lymphocytes Recirculating to the Thymus Suppress the Development of Their Precursors'. *Nature Immunology*, 16(6), pp. 628–634. DOI: 10.1038/ni.3150.

Thomas, S.Y., Banerji, A., Medoff, B.D., Lilly, C.M. and Luster, A.D. (2007) 'Multiple Chemokine Receptors, Including CCR6 and CXCR3, Regulate Antigen-Induced T Cell Homing to the Human Asthmatic Airway'. *The Journal of Immunology*, 179(3), pp. 1901–1912. DOI: 10.4049/jimmunol.179.3.1901.

Tilloy, F., Treiner, E., Park, S.H., Garcia, C., Lemonnier, F., de la Salle, H., Bendelac, A., Bonneville, M. and Lantz, O. (1999) 'An Invariant T Cell Receptor Alpha Chain Defines a Novel TAP-Independent Major Histocompatibility Complex Class Ib-Restricted Alpha/Beta T Cell Subpopulation in Mammals.' *The Journal of Experimental Medicine*, 189(12), pp. 1907–21. DOI: 10.1084/jem.189.12.1907.

Tomura, M., Yu, W.G., Ahn, H.J., Yamashita, M., Yang, Y.F., Ono, S., Hamaoka, T., Kawano, T., Taniguchi, M., Koezuka, Y. and Fujiwara, H. (1999) 'A Novel Function of Valpha14+CD4+NKT Cells: Stimulation of IL-12 Production by Antigen-Presenting Cells in the Innate Immune System.' *Journal of Immunology (Baltimore, Md.: 1950)*, 163(1), pp. 93–101. DOI: ji_v163n1p93 [pii].

Treiner, E., Duban, L., Bahram, S., Radosavljevic, M., Wanner, V., Tilloy, F., Affaticati, P., Gilfillan, S. and Lantz, O. (2003) 'Selection of Evolutionarily Conserved Mucosal-Associated Invariant T Cells by MR1'. *Nature*, 422(6928), pp. 164–169. DOI: 10.1038/nature01433.

Tuovinen, H., Pekkarinen, P.T., Rossi, L.H., Mattila, I. and Arstila, T.P. (2008) 'The FOXP3+ Subset of Human CD4+CD8+ Thymocytes Is Immature and Subject to Intrathymic Selection'. *Immunology and Cell Biology*, 86(6), pp. 523–529. DOI: 10.1038/icb.2008.36.

Uehara, S., Hayes, S.M., Li, L., El-Khoury, D., Canelles, M., Fowlkes, B.J. and Love, P.E. (2006) 'Premature Expression of Chemokine Receptor CCR9 Impairs T Cell Development'. *The Journal of Immunology*, 176(1), pp. 75–84. DOI: 10.4049/jimmunol.176.1.75.

Ussher, J.E., Bilton, M., Attwod, E., Shadwell, J., Richardson, R., de Lara, C., Mettke, E., Kurioka, A., Hansen, T.H., Klenerman, P. and Willberg, C.B. (2014) 'CD161++CD8+ T Cells, Including the MAIT Cell Subset, Are Specifically Activated by IL-12+IL-18 in a TCR-Independent Manner'. *European Journal of Immunology*, 44(1), pp. 195–203. DOI: 10.1002/eji.201343509.

Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M. and Stockinger, B. (2006) 'TGFβ in the Context of an Inflammatory Cytokine Milieu Supports de Novo Differentiation of IL-17-Producing T Cells'. *Immunity*, 24(2), pp. 179–189. DOI: 10.1016/j.immuni.2006.01.001.

De Vries, J.E. (1998) 'The Role of IL-13 and Its Receptor in Allergy and Inflammatory Responses'. *Journal of Allergy and Clinical Immunology*, 102(2), pp. 165–169. DOI: 10.1016/S0091-6749(98)70080-6.

Walsh, D., McCarthy, J., O'Driscoll, C. and Melgar, S. (2013) 'Pattern Recognition Receptors-Molecular Orchestrators of Inflammation in Inflammatory Bowel Disease'. *Cytokine and Growth Factor Reviews*, 24(2), pp. 91–104. DOI: 10.1016/j.cytogfr.2012.09.003.

Wang, J., Ioan-Facsinay, A., van der Voort, E.I.H., Huizinga, T.W.J. and Toes, R.E.M. (2007) 'Transient Expression of FOXP3 in Human Activated Nonregulatory CD4+ T Cells'. *European Journal of Immunology*, 37(1), pp. 129–138. DOI: 10.1002/eji.200636435.

Wilgenburg, B. van. *et al.* (2016) 'MAIT Cells Are Activated during Human Viral Infections'. *Nature Communications*, 7, p. 11653. DOI: 10.1038/NCOMMS11653.

Witt, C.M. and Robey, E.A. (2004) 'The Ins and Outs of CCR7 in the Thymus'. *Journal of Experimental Medicine*, 200(4), pp. 405–409. DOI: 10.1084/jem.20041110.

Wu, L., Antica, M., Johnson, G.R., Scollay, R. and Shortman, K. (1991) 'Developmental Potential of the Earliest Precursor Cells from the Adult Mouse Thymus'. *Journal of Experimental Medicine*, 174(6), pp. 1617–1627. DOI: 10.1084/jem.174.6.1617.

Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A.D., Stroud, J.C., Bates, D.L., Guo, L., Han, A., Ziegler, S.F., Mathis, D., Benoist, C., Chen, L. and Rao, A. (2006) 'FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT'. *Cell*, 126(2), pp. 375–387. DOI: 10.1016/j.cell.2006.05.042.

Wucherpfennig, K.W., Gagnon, E., Call, M.J., Huseby, E.S. and Call, M.E. (2010) DOI: 10.1101/cshperspect.a005140.

Yang, J. and Yan, H. (2017) 'TLR5: Beyond the Recognition of Flagellin'. *Cellular &Amp; Molecular Immunology*, 14, p. 1017. DOI: 10.1038/cmi.2017.122.

Yang, X.O., Panopoulos, A.D., Nurieva, R., Seon, H.C., Wang, D., Watowich, S.S. and Dong, C. (2007) 'STAT3 Regulates Cytokine-Mediated Generation of Inflammatory Helper T Cells'. *Journal of Biological Chemistry*, 282(13), pp. 9358–9363. DOI: 10.1074/jbc.C600321200.

Yang, Y. and Wilson, J.M. (1996) 'CD40 Ligand-Dependent T Cell Activation: Requirement of B7-CD28 Signaling through CD40.' *Science (New York, N.Y.)*, 273(5283), pp. 1862–1864. DOI: 10.1126/science.273.5283.1862.

Yewdell, J.W., Bennink, J.R. and Hosaka, Y. (1988) 'Cells Process Exogenous Proteins for Recognition by Cytotoxic T Lymphocytes.' *Science (New York, N.Y.)*, 239(4840), pp. 637–40. DOI: 10.1126/science.3257585.

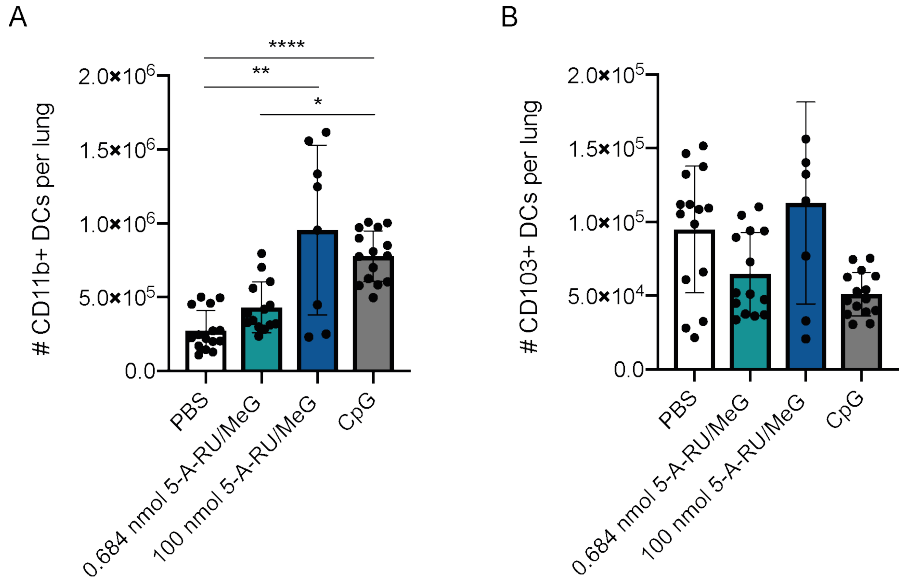
Yoshie, O. and Matsushima, K. (2015) 'CCR4 and Its Ligands: From Bench to Bedside'. *International Immunology*, 27(1), pp. 11–20. DOI: 10.1093/intimm/dxu079.

Yoshimoto, T., Takeda, K., Tanaka, T., Ohkusu, K., Kashiwamura, S., Okamura, H., Akira, S. and Nakanishi, K. (1998) 'IL-12 up-Regulates IL-18 Receptor Expression on T Cells, Th1 Cells, and B Cells: Synergism with IL-18 for IFN-Gamma Production.' *Journal of Immunology (Baltimore, Md.: 1950)*, 161(7), pp. 3400–3407. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9759857.

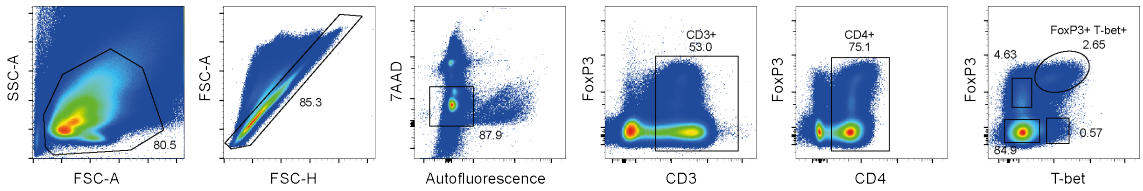
Zabel, B.A., Agace, W.W., Campbell, J.J., Heath, H.M., Parent, D., Roberts, A.I., Ebert, E.C., Kassam, N., Qin, S., Zovko, M., LaRosa, G.J., Yang, L.L., Soler, D., Butcher, E.C., Ponath, P.D., Parker, C.M. and Andrew, D.P. (1999) 'Human G Protein-Coupled Receptor GPR-9-6/CC Chemokine Receptor 9 Is Selectively Expressed on Intestinal Homing T Lymphocytes, Mucosal Lymphocytes, and Thymocytes and Is Required for Thymus-Expressed Chemokine- Mediated Chemotaxis'. *Journal of Experimental Medicine*, 190(9), pp. 1241–1255. DOI: 10.1084/jem.190.9.1241.

Zhu, J., Guo, L., Watson, C.J., Hu-Li, J. and Paul, W.E. (2001) ‘Stat6 Is Necessary and Sufficient for IL-4’s Role in Th2 Differentiation and Cell Expansion’. *The Journal of Immunology*, 166(12), pp. 7276–7281. DOI: 10.4049/jimmunol.166.12.7276.

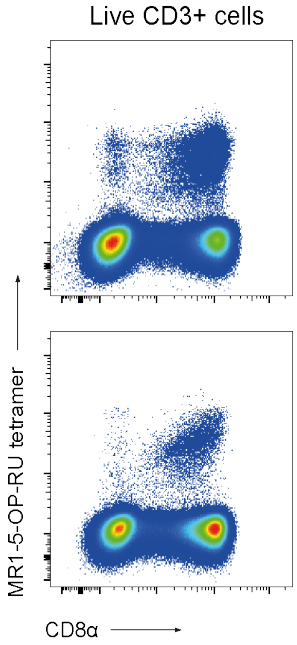
Appendix



Appendix Figure 1 – DC numbers in the lungs of C57BL/6 mice after intratracheal injection of PKH26 and followed stimulation with PBS, 5-A-RU/Meg or CpG. (A) $CD11b^+$ DC numbers. (B) $CD103^+$ DC numbers.



Appendix Figure 2 – Complete gating strategy of $FoxP3^+$ $T\text{-}bet^+$ T cells in human thymus. Thymocytes were gated for single, live and autofluorescent$^-$ cells. They were further gated on $CD3^+$ and $CD4^+$ cells and analyzed for FOXP3 and T-bet expression.



Appendix Figure 3 – MR1-5-OP-RU tetramer staining against CD8α in human PBMCs. PBMCs were previously gated on single, live $CD3^+$ cells. In this figure representative plots of 2 different donors are shown.