TÉCNICO LISBOA

The Role of MicroRNA-181a in the Development and Differentiation of Pro-Inflammatory γδ T Cell Subsets

Marta Gaspar Rodrigues da Silva

Thesis to obtain the Master of Science Degree in

Biomedical Engineering

Supervisors: Prof. Dr. Anita Raquel Quintal Gomes
Prof. Dr. Cláudia Alexandra Martins Lobato da Silva

Examination Committee

Chairperson: Prof. Dr. Maria Margarida Fonseca Rodrigues Diogo
Supervisor: Prof. Dr. Anita Raquel Quintal Gomes
Member of the Committee: Prof. Dr. Ana Sofia Bregieiro Eulálio

September 2021

Preface

The work presented in this Thesis was performed at Bruno Silva-Santos Lab, Instituto de Medicina Molecular João Lobo Antunes of Faculdade de Medicina da Universidade de Lisboa (Lisbon, Portugal), from September 2020 until July 2021, under the supervision of Prof. Dr. Anita Gomes and Prof. Dr. Bruno Silva-Santos. The Thesis was co-supervised at Instituto Superior Técnico by Prof. Dr. Cláudia Lobato da Silva.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Acknowledgments

I would like to express my sincere gratitude to my iMM supervisors, Bruno Silva-Santos and Anita Q. Gomes, for giving me this opportunity and for supervising my work. I also want to thank my co-supervisor at Técnico, Cláudia Lobato da Silva, for being such a great professor and mentor. I want to thank Daniel Inácio as well for all the hours we spent working on experiments and for all of the advice and guidance. I must also thank everyone in the Lab and at the iMM facilities for being so inspiring and helpful.

To my parents, for always being so loving and supportive. To my big brother, for always watching (over me) — pun intended. To my grandparents, my aunts and uncles, and my cousins, for the amazing environment they provided me growing up. To Manel, for making me laugh through the good, the bad, and the worst, and for being my safe haven. To Marta, for being the complete opposite of me and a huge beam of light in my life. To the rest of my friends, and in alphabetical order so no one gets jealous, Ana, Chica, Coelho, David, Dinis, Fi, Gui F., Guizão, Gusmão, Orvalho, Pedroca, Pombal, Ruizinho, Silveira, Sofs, Trindade, Torrinha, Valente, Vasco B. and Vasco L., for making life so much fun.

Abstract

γδ T cells exert their immuno-(patho)physiological functions mainly through the secretion of two pro-inflammatory cytokines: interleukin-17A and interferon-γ. These are produced by γδ T cell subpopulations (γδ17 and γδIFN) that differentially respond to TCR signal strength and are pre-programmed in the thymus, migrating subsequently to the periphery. MicroRNAs negatively regulate gene expression, modulating T cell function. miRNA-181a is a rheostat of thymic TCR sensitivity, affecting T cell activation, thus making it a potential modulator of γδ T cell functions. Preliminary data showed distinct expression levels of miR-181a in γδ T cell subpopulations throughout development. Therefore, by studying miR-181a-deficient vs. -sufficient mice, we aimed at dissecting the role of miR-181a in the development and differentiation of pro-inflammatory of γδ T cell subsets. *Ex vivo* analysis of lymphoid organs revealed that the fetal thymus of miR-181a-deficient mice exhibited a decreased frequency of γδ17 cells, with neonatal and adult organs additionally showing an increased frequency of γδIFN cells, compared to WT controls. Cells lacking in miR-181a showed a slight increase in calcium flux, and upon *in vitro* stimulation with αCD3/28 showed decreased frequency of IFN-γ^+ cells. A bioinformatical target search identified several promising mRNA targets associated with the γδ17 and γδIFN machinery. This work highlights a modulatory role for miR-181a in γδ T cell development and differentiation, possibly by regulating TCR signaling strength, steering the commitment of γδ T cells into a γδ17 fate early in the thymus, while suppressing the γδIFN fate, and potentially impacting on peripheral γδIFN T cells.

Keywords

miR-181a; γδ T cells; thymic development; effector T cell differentiation; cytokines; IL-17; IFN-γ; TCR signaling

Resumo

As células T γδ exercem funções imuno-(pato)fisiológicas através da secreção das duas citocinas pró-inflamatórias: interleucina-17A e interferão-γ. Estas são produzidas por subpopulações de células T γδ que respondem diferencialmente à estimulação do TCR, estando pré-programadas no timo e migrando subsequentemente para a periferia. O MicroRNA-181a é um pequeno RNA não codificante que atua que atua como reóstato à sensibilidade do TCR no timo, participando na ativação de células T αβ. Estudos preliminares mostraram níveis distintos de expressão do miR-181a em subpopulações de células T γδ ao longo do desenvolvimento. Assim, neste estudo, recorremos a ratinhos miR-181a *knockout* e *wild-type* para decifrar o papel do miR-181a no desenvolvimento e diferenciação das células T γδ. A análise *ex vivo* de orgãos linfóides revelou que o timo embriónico de ratinhos miR-181a$^{-/-}$ têm menor frequência de células γδ17, tendo-se observado uma maior frequência de células γδIFN, em comparação com ratinhos WT. Células sem o miR-181a têm menor capacidade de mobilizar cálcio para o seu citosol, e estimulação *in vitro* com αCD3/28 leva a uma menor frequência de células T γδ produtoras de IFN-γ Através de uma análise bioinformática, identificamos vários mRNA alvo promissores associados aos mecanismos de diferenciação das células γδ17 e γδIFN. O nosso trabalho revela um papel modelador do miR-181a no desenvolvimento e diferenciação das células T γδ, possivelmente através da mediação da força de sinalização do TCR, promovendo o *commitment* ao programa γδ17 no timo, enquanto o programa γδIFN é suprimido, e possivelmente afetando as células γδIFN na periferia.

Palavras Chave

miR-181a; células T γδ; desenvolvimento; diferenciação de células T; IL-17; IFN-γ; sinalização de TCR

Contents

List of Figures

List of Tables

Acronyms

AGO	Argonaute
APC	Antigen Presenting Cell
bp	Base Pair
BM	Bone Marrow
CTL	Cytotoxic T Lymphocyte
CTV	CellTrace Violet
D	Diversity
DC	Dendritic Cell
DETC	Dendritic Epidermal T cell
DGCR8	DiGeorge syndrome critical region
DN	Double Negative
DSS	Dextran Sulfate Sodium
DP	Double Positive
DP	Double Producers
EAE	Experimental Autoimmune Encephalomyelitis
Erg	Early growth response
ERK	Extracellular Signal-Regulated Kinase
eIF4F	Eukaryotic Initiation Factor 4F Complex
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FTOC	Fetal Thymic Organ Cultures
GRN	Gene Regulatory Network

HMG High-Motility Group

HSC Hematopoietic Stem Cell

IBS Inflammatory Bowel Syndrome

iCLIP individual-nucleotide resolution UV Cross-Linking and Immunoprecipitation

IEL Intraepithelial Lymphocyte

IFN Interferon

IL Interleukin

iNKT Invariant Natural Killer T Cell

ITAM Immunoreceptor Tyrosine-based Activation Motif

ISP Immature Single Positive

J Joining

KO Knockout

mAb Monoclonal Antibody

MAP Microtubule Associated Protein

MAPK Mitogen-Activated Protein Kinase

MFI Mean Fluorescence Intensity

MHC Major Histocompatibility Complex

miR MicroRNA

miRISC MicroRNA-Induced Silencing Complex

miRNA MicroRNA

mRNA Messenger RNA

MS Multiple Sclerosis

NEAA Nonessential Amino Acids

NK Natural Killer

NKT Natural Killer T Cell

NF-κB Nuclear Factor-κB

TCR T Cell Antigen Receptor

TdT Terminal Deoxynucleotidyl Transferase

TF Transcription Factor

Th T helper cell

TNF Tumor Necrosis Factor

Treg Regulatory T Cell

PABPC Poly(A)-Binding Protein

PAMP Pathogen-Associated Molecular Pattern

PBMC Peripheral Blood Mononuclear Cell

PBS Phosphate Buffer Saline

PCR Polymerase Chain Reaction

PI3K Phosphoinositide 3-Kinase

pLN Peripheral Lymph Node

pre-miRNA pre-microRNA

pri-miRNAs Primary microRNAs

PRR Pattern-Recognition Receptors

RIP-seq RNA Immunoprecipitation Sequencing

RT-qPCR Real-Time Quantitative PCR

scRNA-seq Single Cell RNA sequencing

SFK SRC-family kinases

SP Single Positive

STAT Signalling Transducer and Activator of Transcription

UTR Untranslated Region

V Variable

WT Wild-Type

1

Introduction

Contents

1.1 Innate and Adaptive Immunity

The immune system plays an important role in the defense against foreign agents, like bacteria, fungi, viruses and chemicals, and it also has an outstanding role in the physiology of other systems. It is composed of a complex network of cells with differentiating potential, which can convert external stimuli into complex intracellular regulatory networks [1, 2].

Mammalian immunity possesses three important characteristics: (a) the ability to recognise and mount a response against thousands of different pathogens, while usually not reacting against endogenous agents, i.e. self-antigens, which would lead to autoimmunity [3]; (b) the capacity to act systemically, away from the starting site of the immune response; and (c) the ability of the immune response to be amplified and controlled by feedback mechanisms that make it effective, but not exacerbated [2].

The immune system can be subdivided into two types of immunity — the innate immunity, and the adaptive immunity. While innate immunity comes into play within the first hours or days after the invasion of an outside agent, thus constituting the first line of defense against external microorganisms and pathogens, adaptive immunity is essential in the later times of an infection, occurring through the recognition of specific antigens. Regarding the cells involved in each of these two processes, we can include phagocytes (e.g. neutrophils and macrophages), dendritic cells (DCs), natural killer (NK) cells and other innate lymphoid cells, and mast cells as the major players of innate immunity, while lymphocytes are the key effector cells in adaptive immunity.

Another relevant difference is that innate immune cells can directly recognise microorganisms through germline-encoded pattern-recognition (PRRs) receptors, which recognize and bind to conserved molecular motifs of pathogens called pathogen-associated molecular patterns (PAMPs). PRRs are able to discern between groups of related microbes, but are not so specific as to be able to differentiate between microbes within the same family. The adaptive immune response, on the other hand, is much more specific and diverse than the innate response, as lymphocytes have membrane receptors that recognize subtle differences between a large variety of antigens [4]. Through the process of clonal selection and expansion, lymphocytes with the same specificity recognise a given antigen and get activated, which is followed by their proliferation [5]. These cells are able to retain memory of a first infection, and mount a faster and heightened response to subsequent ones [6], while retaining a tolerance for self-antigens, thus preventing autoimmunity [7].

Adaptive immunity can be further subdivided into humoral immunity, mediated by antibodies secreted by B lymphocytes, and cell-mediated immunity, mediated by T lymphocytes. While antibodies target extracellular microbes and eliminate them through phagocytes, T lymphocytes kill microbes that survive within phagocytes, as well as intracellular microbes replicating within transformed cells. Within T cells, there are many subtypes, which express different T cell receptors (TCRs) or cell surface proteins. Specifically, T lymphocytes expressing the α and β chains are called $\alpha\beta$ T cells. Depending on whether

these express CD4, CD8 or NK1.1, they are called CD4, CD8 or Natural Killer (NKT) T cells. CD4 T cells, in particular, are considered important mediators of adaptive immunity, and take on several roles, such as enhancing and maintaining CD8 T cell response and regulating the immune response during defense against pathogens or autoimmunity [8]. On the other hand, if the T lymphocytes express the γ and δ chains, they are classified as $\gamma\delta$ T cells [2]. These are the main focus of this study and will be described in detail in the next sections.

1.2 $\gamma\delta$ T cell Biology

1.2.1 $\gamma\delta$ T cell biogenesis

Mammal Hemapoietic Stem Cells (HSCs) residing in the fetal liver and the bone marrow (BM) seed the thymus. Here, they become immature T cells that can undergo series of changes that turn them into different types of mature T cells. Most of these cells are double negative (DN) for both the CD4 and CD8 markers and some of them will go on to become other cell types, like NK cells. The remaining DN cells either commit to an $\alpha\beta$ or to a $\gamma\delta$ lineage and ultimately acquire effector functions [9]. Cells from both of these lineages undergo a somatic rearrangement of their TCR to acquire effector functions, and they eventually express CD3 and the ζ proteins, which, along with either the $\alpha\beta$TCR or the $\gamma\delta$TCR, will be fundamental for surface expression [2]. This somatic rearrangement, or V(D)J recombination, is a process of site-specific gene rearrangement of the variable (V) gene segments with the diversity (D) and joining (J) gene segments of individual lymphocytes [2, 10], and it is greatly responsible for the vast variability of TCRs.

After this stage, $\alpha\beta$ T cells will become immature single positive (ISP) for CD4, ISP for CD8, or double positive (DP) for CD4 and CD8. These ISP cells usually end up becoming DP cells as well. DP cells then go through positive selection, a process in which they are presented with self-antigen-Major Histocompatibility Complex (MHC) complexes by antigen presenting cells (APCs). Those that interact with MHC-II or MHC-I molecules are positively selected into mature $CD4^+$ and $CD8^+$ single positive (SP) cells, respectively, whereas the ones that fail to do so suffer apoptosis. This allows the surviving cells to identify foreign peptides displayed by the MHC molecules, thus making them effective in an immune response context. After this, the surviving SP cells, along with some DP cells, suffer negative selection, in which they are again presented with self-antigen-MHC complexes. This time around, the cells that bind too strongly to those complexes are the ones that suffer apoptosis. This serves to protect the immune system against autoimmunity [2, 11–13]. From this point onward, $\alpha\beta$ T cells move to secondary lymphoid tissues, where they gain effector functions upon an inflammatory trigger [8].

In contrast, most $\gamma\delta$ T cells acquire their effector functions in the thymus, and afterwards they move mostly to non-lymphoid tissues, such as the intestine, the reproductive tract or the skin [14–16]. In

these tissues, they represent a significant portion of all T cells present ($\sim$ 50%); in the circulating blood, however, they represent up to 1% of all T cells available. [17, 18]. Importantly, the γδTCR is the first TCR to be expressed in mice, as well as in other vertebrates, and it consists of a TCRγ and a TCRδ chain. In the case of γδ T cells, the V(D)J recombination is an ordered arrangement that leads to a timed production of specific γδ subsets that populate different tissues, thus defining developmental waves (see Figure 1.1) [19–21]. Of note, in the current work we will use the Heilig and Tonogawa system to refer to Vγ gene nomenclatures [22].

The first subset of γδ T cells — and, notably, the first subset of T cells —, are produced in the fetal thymus and express an invariant Vγ5Vδ1 TCR [23,24]. These cells, known as dendritic epidermal T cells (DETCs), arise at around the fourteenth embryonic day, during which terminal deoxynucleotidyl transferase (TdT) — important for junctional diversity by adding nucleotides at the 3'-ends of gene segments during V(D)J recombination — is not yet expressed, making them invariant [25]. Afterwards, these cells home to the skin, where they self-renew throughout life to maintain their homeostatic density [26]. They aid in wound healing and immune surveillance [27]. The second wave of γδ T cells are produced in the fetal thymus as well, but they express the Vγ6 chain [28]. They then home to epithelial sites, like the uterus, the tongue, the lungs, and the meninges [15, 29]. Similarly to DETCs, they are produced during gestation, where TdT expression is lacking, which means that they are invariant as well. Right after, Vγ7$^+$ cells appear and become intestinal intraepithelial lymphocytes (IELs) [30]. Finally, the last γδ T cells produced express the Vγ4 and Vγ1 chains, and they do so towards the end of gestation and after birth, throughout adulthood [15]. Most of these cells will go on to populate lymphoid tissues, such as the spleen and the lymph nodes; here, they represent the majority of γδ T cells in adult mice. Because they are produced when TdT expression is present, they possess higher junctional diversity than other γδ T cell subsets. During this perinatal phase, cells expressing a semi-invariant Vγ1Vδ6 TCR are formed. These are also referred to as NKTγδ cells because they express the NK marker NK1.1 and have a memory innate-like phenotype [31–33].

The repertoire of antigens that γδ T cells are able to recognize is more limited than that of αβ T cells [14, 34], but they have been reported to participate in tumor surveillance [35, 36], and in the response against microbial pathogens and viruses [37, 38]. Moreover, the fact that γδ T cells mature in the thymus and then home to specific tissues already endowed with a pro-inflammatory phenotype makes them very fast in initiating effector functions at those sites. There, they are able to act as a first line of defense, based on "innate-like" characteristics. On the other hand, their orchestrated developmental stages in the thymus, as well as the heterogeneity of subsets based on their γδTCR expression, give γδ T cells some adaptive-like characteristics. Altogether, the role of γδ T cells seems to sit between the innate and the adaptive immune system, also referred to as "adaptate" immunity [15, 39–41].

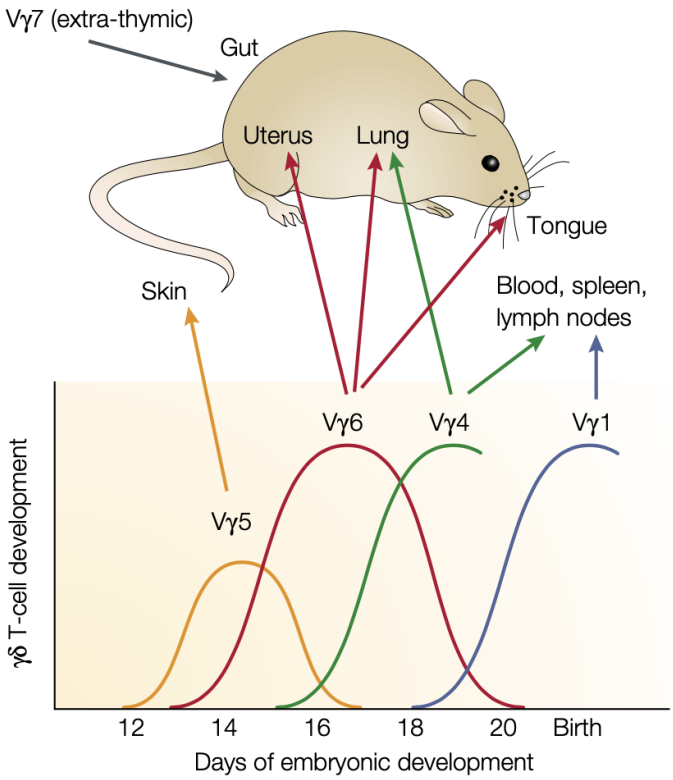


Figure 1.1: Mouse γδ T cell generation is developmentally programmed. γδ T cells bearing T Cell Receptors that are encoded by specific Vγ-gene segments (see graph) are exported from the thymus at defined periods of fetal and neonatal development, and then migrate to and populate different epithelial-rich tissues in adult animals. The development of some Vγ7^{+} intestinal intraepithelial lymphocytes might be thymic independent. From [14].

1.2.2 Pro-inflammatory γδ T cell subsets

The effector functions of γδ T cells are mostly associated with the production of two cytokines: Interferon-γ (IFN-γ) and Interleukin-17A (simplified to IL-17). IFN-γ-producing γδ T cells (γδIFN) are important in the defense against viral and intracellular bacterial infections (e.g. *Listeria monocytogenes*) [42], as well as against cancer cells [35]. IL-17-producing γδ T cells (γδ17) have been associated in the early stages of defense against several extracellular pathogens, such as *Escherichia coli*, *Candida albicans* and *Staphylococcus aureus* [43]. However, overproduction of either of these cytokines can lead to several complications. For the former, it can mean an exacerbated reaction to severe malaria [44], and for the latter it can lead to a higher susceptibility to autoimmune conditions, e.g. psoriasis and the animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE) [43, 45, 46].

Pro-inflammatory cytokine production of γδ T cells associates, in different developmental waves, to certain Vγ populations (see Table 1.1). In an early developmental phase, the first wave is marked by IFN-γ production associated to Vγ5^{+} cells — between E13 and E16 —, which populate the epidermis

of the skin. A second wave appears, this time associated to IL-17 production by Vγ6$^+$ cells — between E14 and E20 —, which populate several epithelial tissues, and later in gestation, to Vγ4 — from E16 onwards —, which home mostly to lymphoid tissues, like the spleen or lymph nodes. Towards the end of gestation, after birth and throughout adulthood, a wave marked by IFN-γ provision is correlated to Vγ1 and Vγ7 — from E18 onwards —, which also mainly home to lymphoid tissues [43, 47–50]. It is noteworthy that although Vγ1-expressing cells are mainly IFN-γ producers, they are known to be able to express small amounts of IL-17 as well [51]. The Vγ1$^+$ population is marked by the secretion of IFN-γ, IL-4 and IL-15 [31]. Moreover, a seemingly naive population of γδ T cells emerges during the neonatal period, which produce IFN-γ and that polarize peripheral tissues [52, 53].

Table 1.1: Development, homing, and cytokine profiles of mouse γδ T cell subsets. Adapted from [43].

Subset	Thymic development	Tissue distribution	Cytokines
Vγ1$^+$	E18 and beyond	Lymphoid tissue, liver, lung	IFN-γ (minor producers of IL-17)
Vγ4$^+$	E18 and beyond	Lymphoid tissue, lung, liver, dermis	IL-17 and IFN-γ
Vγ5$^+$	E13-E16	Epidermis	IFN-γ
Vγ6$^+$	E14-E20	Uterus, lung, tongue, liver, placenta, kidney	IL-17
Vγ7$^+$	E18 and beyond	Intestinal intraepithelial layer	IFN-γ

1.2.2.A Cell Surface Markers

In order to effectively study and distinguish between the effector subsets of γδ T cells, it is important to have cell markers to identify and analyze them (see Figure 1.2). There are many identified cell surface markers useful for the identification of γδ T cell subsets. We will focus on the most relevant ones for our study in the following paragraphs.

A meaningful discovery in this field was made in the host lab by Ribot et al. and colleagues, that identified CD27 as a surface marker able to distinguish between IFN-γ- and IL-17-producing γδ T cells [49]. CD27 belongs to the tumor necrosis factor (TNF) superfamily and, prior to this study, it was already being used for the identification of αβ and NK T cells [54, 55]. Using CD27 expression, γδ T cells can be categorized into two groups: CD27$^-$, also referred to as γδ27$^-$, who are mainly IL-17 producers, but can be induced to co-express both IL-17 and IFN-γ under inflammatory conditions [56], and CD27$^+$, or γδ27$^+$, which tend to be IFN-γ producers and represent the most significant proportion of all γδ T cells ($\sim$ 70 – 90%) in steady-state in the spleen, lymph nodes, lungs and gut.

Another seminal work concerning not only effective cell markers to distinguish between the two γδ T cell subsets, but also their overall thymic development, was published by Nital et al. [57]. The authors identified the earliest γδ T cell populations in thymus to progress from being immature CD25$^+$CD24$^+$, to CD25$^-$CD24$^+$, and finally to mature CD25$^-$CD24$^-$. From this point onwards, the subsets arising from this population are all CD24$^-$. The subsequently produced IL-17-committed γδ T cells are characterized as

being CD44^{hi}CD45RB^{-}, whereas the IFN-γ-committed ones are characterized as being CD45RB^{+}. The IFN-γ "pathway" follows the CD44^{-}CD45RB^{-}, to CD44^{-}CD45RB^{+}, to CD44^{+}CD45RB^{+} progression, but CD44^{-}CD45RB^{-} cells can commit to either one of the two pro-inflammatory γδ T cell subsets.

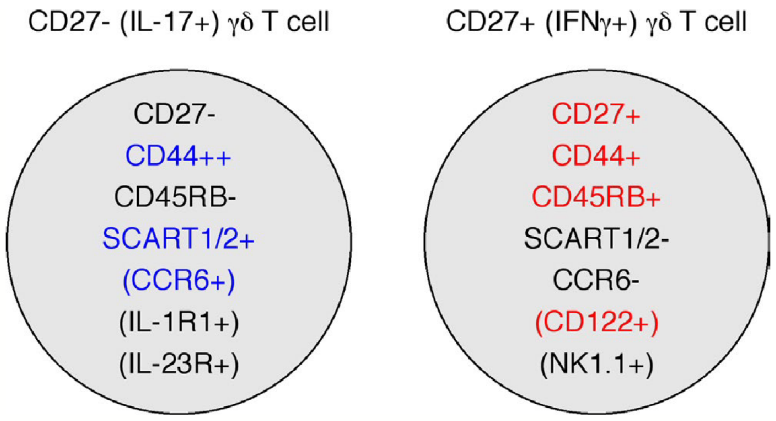


Figure 1.2: Expression status of cell surface receptors that segregate effector γδ T cell subsets. IL-17^{+} IFN-γ^{+} γδ T cell subsets express different surface receptors enabling their identification and isolation based on these markers. The most commonly used markers are highlighted for IL-17^{+} γδ T (blue) and IFN-γ^{+} γδ T cells (red). Noted within brackets are surface markers expressed on particular subpopulations of the respective effector subsets. From [15].

1.2.2.B TCR signaling strength and γδ T cell effector fate

The γδTCR complex is made of not only the γδTCR itself, but also of several interacting CD3 chains. In mice, it has the following stoichiometry: $TCR\gamma\delta CD3_{\epsilon 2\gamma 2\zeta 2}$ [58].

Intracellular TCR signaling begins when the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domain of CD3 subunits become phosphorylated by co-clustering of the TCR and SRC-family kinases (SFKs) — Lck and Fyn [59, 60]. In αβ T cells, MHC-peptide complexes in APCs interacting with the TCR lead to TCR signaling and activation of co-stimulatory signaling receptors, such as CD28, prompting the recruitment of the CD8 or CD4 co-receptors, which in turn bind to regions of the MHC I or II, respectively. Intracellular domains of CD8 or CD4 then bind to SFKs, causing phosphorylation of ITAMs. In γδ T cells, however, the recruitment of SFKs to the TCR complex and the interaction with the MHCs is still unknown [61, 62].

After phosphorylation of ITAMs within the CD3 subunit, the tyrosine kinase Zap70 is recruited to to the TCR, and subsequently phosphorylated and activated, thus leading to the phosphorylation of scaffolding proteins, one of which is LAT. This in turn produces docking sites for the recruitment of other adaptor molecules, as well as the recruitment and activation of other signaling molecules, which include the phospholipase PLCγ1. These events generate a LAT signalosome, which triggers the propagation of several downstream signaling events. The propagation of downstream signaling events caused by the

formation of the LAT signalosome is done through three major signaling pathways: the nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) and the Ca^{2+}-calcineurin signaling pathways. NF-κB signaling leads to nuclear translocation of the NF-κB and REL transcription factors (TFs), MAPK signaling leads to actin polymerization and activation of several TFs, and Ca^{2+}-calcineurin leads to nuclear translocation of NFAT. The combined activity of all these players results in T cell proliferation, migration, cytokine production and exertion of effector function [60].

The notion that the TCR and its signaling strength have a considerable impact on γδ T cell differentiation has been a subject of interest for some time [15, 16]. There are theories postulating that the TCR might play a role in the bifurcation into either the αβ or the γδ T cell lineages; in particular, there is some evidence pointing to the commitment to the γδ lineage requiring that DN cells receive stronger TCR signaling [63, 64]. Then, during effector function acquisition, the fact that certain Vγ populations preferentially secrete either one of the two pro-inflammatory cytokines corroborates the theory that the γδTCR somehow influences such a bias [48]. Specifically, a strong TCR signal has been pinpointed to induce an IFN-γ effector potential and, in opposition, a weak TCR signal to promote an IL-17 one.

In favor of the theory postulating that TCR signaling strength influences the commitment to either an αβ or a γδ fate are the increased number of signaling events downstream of γδTCR, when compared to the αβTCR [65]. How DN cells differentiate between TCR signal strengths has not been completely elucidated, but it is thought that the extracellular signal-regulated kinase (ERK) pathway plays an important role. In particular, the activation of this pathway leads to the activation of early growth response (Erg) TFs, which in turn inhibits Id3, which promotes the γδ fate [66]. Notably, this mechanism is mediated by DBP, of the ERK pathway, in which it enables ERK to bind a set of proteins required for the γδ T cell lineage, which prolongs the ERK signal [67].

It has been reported that IFN-γ-associated γδ T cells require thymic expression of their TCR antigen to differentiate into the IFN-γ lineage [68]. Consistently, IFN-γ-producing populations, like DETCs and γδNKT cells, also use this mechanism to differentiate [32]. For instance, the γδ skin-residing-IFN-γ-producing population, DETCs, induce the Erg3 pathway through Skint-1^+ cell engagement, which promotes IFN-γ production, while suppressing genes associated with the IL-17 machinery, like Sox13 and RORγt [69, 70]. Muñoz et al. showed that a selective defect in the surface expression of the γδTCR (CD3DH mice) led to a weaker TCR signal, which resulted in a reduced number of γδ thymocytes. Also, both pro-inflammatory subsets had a lower frequency in embryos. However, although this observation remains consistent into adulthood for IFN-γ-producing γδ T cells, that is not the case for IL-17-producing T cells, as cell frequency became similar to that of wild-type mice between the first and the sixth weeks of age [50]. Using the FTOC system and by adding the anti-TCRδ antibody GL3 to E15 thymic lobes, which boosts TCR signal strength, Sumaria et al. confirmed that there was a significant increase in the number of $CD44^+CD45RB^+$ cells (IFN-γ producers) at the expense of γδ17 cells, which exhibited reduced

numbers [57]. The difference was not only observed in terms of absolute cell numbers, but also in terms of cytokine production, in a dose-dependent fashion. The subsequent inhibition of the ERK/Microtubule Associated Protein (MAP) pathway confirmed that this pathway directly mediated TCR signaling and affected cytokine production by γδ T cells. Indeed, differential gene expression analysis has shown that, in immature CD24$^-$ γδ T cells, there are clusters expressing higher levels of TCR signaling genes, such as *Nfact1* and *Prkch*, and clusters expressing higher levels of γδ17-associated genes, such as *Sox13* and *Blk*. It can thus be hypothesized that the IFN-γ "pathway" is dependent on signal strength, and that cells belonging to the IL-17 "pathway" are already primed towards that fate prior to maturation [53].

Although several studies seem to suggest that, in order for γδ T cells to follow an IL-17 fate, a weak TCR signal is required [50, 57, 69, 70], some studies contradict this notion. Of note, CD73 — a marker associated with strong TCR engagement —, is expressed on adult thymic γδ17 cells [71]. Additionally, mice carrying a mutation that almost impairs the function of the TCR-proximal signaling kinase Zap-70 show average numbers of total γδ T cells, but an almost full depletion of γδ17 cells [72].

In conclusion, it seems clear that strong TCR signaling steers γδ T cells towards an IFN-γ lineage; however, whether or not a weak signal steers them towards an IL-17 fate is not yet apparent.

1.2.2.C Transcriptional landscape of effector γδ T cells

The molecular and transcriptional cues received by γδ T cells affect how they develop and differentiate into either one of their two pro-inflammatory cytokine subsets — γδIFN and γδ17 —, which in turn affects how they respond to external stimuli. For example, IL-2 mediates the survival of both effector γδ T cell subsets upon *Plasmodium berghei* infection, via the CD28 receptor. On the other hand, not only is CD27 an important regulator of overall γδ thymocyte development [49], but also its costimulation with γδTCR leads to peripheral expansion and enhanced responses of IFN-γ-producing (γδ27$^+$) cells upon *P. berghei* infection as well [41, 73].

The αβ T cell counterparts of IFN-γ- and IL-17-expressing γδ T cells are, respectively, Th1 and Th17, part of the T helper CD4$^+$ lineage. They are among the key players in adaptive immunity and, unlike γδ T cells, they acquire their effector functions upon an inflammatory trigger after moving to peripheral lymphoid organs upon thymic development [8]. Understanding the molecular similarities and disparities between these cells and their γδ T cell counterparts is useful to unveil the molecular mechanisms underlying γδ T cell differentiation.

Both IFN-γ-producing γδ T cells and Th1 cells express high levels of T-bet [74]. Not only that, this TF was shown to be required for *in vitro* and *in vivo* IFN-γ production by γδ27$^+$ cells, with T-bet knockout (KO) cells showing marked deficiencies in Th1 cells *in vitro* and *in vivo* [75]. Likely, an additional T-box family member, Eomesodermin (Eomes), also plays a role in increasing IFN-γ expression, promoting Th1 differentiation [76]. On γδIFN cells, however, Eomes has controversial results. On the one hand, its

absence is completely dispensable for the production IFN-γ by γδ T cells, so it is not necessary for γδIFN differentiation [77]; but on the other hand, Eomes$^+$ cells have higher IFN-γ production upon stimulation, increased expression of *Lyc6C* and *Il2rb* and an activated memory phenotype, characterized by the expression of CD44. Additionally, Eomes expression could be induced by IL-12 and IL-4 [78]. In addition to T-bet, TCF1 and the LEF1 promote the IFN-γ "pathway" of γδ T cells, while inhibiting the IL-17 one [79].

The master regulator of Th17 cells is RORγt, a TF overexpressed by a high percentage of activated cells, with cells lacking it producing very little IL-17 [80]. RORγt plays a major role in the differentiation of γδ17 cells as well, with RORγt-deficiency leading to a complete abrogation of IL-17 production by γδ T cells [81]. Additionally, two high-motility group (HMG) box transcription factors — SOX4 and SOX13 — have been identified to be crucial to the gene regulatory network of γδ17 cells [79]. Specifically, SOX4 and SOX13 regulate the expression of *Rorc* and *Blk*, respectively. The *Blk* gene is known to negatively regulate TCRγδ and to be essential for IL-17 production by γδ T cells expressing Vγ6 [82]. However, unlike in Th17 cells, IL-17 production by γδ T cells is independent from BAFT and IRF4 [77, 83]. Instead, both of these TFs are required for the recruitment of γδ17 cells to inflamed tissue during autoimmunity, infection or cancer, as they partake in the downregulation of CCR6, which restricts them from entering non-inflamed tissue [84]. STAT3 has been pinpointed as crucial for the differentiation of Th17 by regulating the expression of RORγt, but it has been shown to be unnecessary for γδ T cell differentiation [85]. Another member of the STAT family, STAT5, is required at a low level to create a balanced state that optimizes both Th1 differentiation and Th17 expansion [8]. Interestingly, a recent study has brought to light the duality of STAT5 in γδ T cell differentiation as well. Of note, mice with a STAT5 conditional deficiency displayed a loss of γδ17 cells in adult organs, showing a higher susceptibility to EAE. Using mutated STAT5 hyperactive mice, STAT5A was shown to promote γδ17 expansion, whereas STAT5B promoted γδIFN expansion [86].

A recent paper established a high resolution map of differentiation of γδ T cells from the fetal and the adult thymus using scRNA-seq, and uncovered some of the temporal regulation leading to the differentiation of γδ17 cells [53]. This study uncovered five gene regulatory networks (GRN) in the fetal thymus, and another one in the adult thymus. The earliest GRN is comprised of genes related to early thymic progenitors, such as *Bcl11a*, *Cd44* and *Kit*. The following network contained genes associated with recombination, such as *Rag1*, *Rag2*, *Pdl4* and *Notch3*. The third network was proliferation-related and included such genes as *Mki67* and *Pcna*. The last two GRNs in the fetal thymus were: (i) γδ17-associated, comprising *Sox13*, *Maf*, *Blk* and *Rorc*, and (ii) γδIFN-associated, comprising *Il2rb*, *S1pr1* and *Klra10*. The additional GRN of the adult thymus was activated at mature γδ17 stages, and some of the genes it encompassed are: *Cd44*, *Icos*, *Il18r1*, *Ccr2* and *Ccr6*. By using KO mice for *Sox13*, *Maf* and *Rorc*, the authors were also able to ascertain that SOX13 acts upstream of c-MAF to activate its expression. Moreover, *Sox13* KO mice upregulated genes related to TCR signaling, hypothesizing that

Sox13 may somehow regulate TCR signaling during early thymic γδ T cell differentiation. Indeed, mice with Sox13-deficient Vγ4$^+$ γδ17 cells showed increased protection to psoriasis-like dermatitis [87].

Besides suffering transcriptional regulation, the development and maintenance of γδ T cells also requires post-transcriptional regulation, which may involve epigenetic mechanisms, such as microRNA-mediated regulation [88], the focus of the following subsection.

1.3 MicroRNAs

MicroRNAs (miRNAs or miRs) are small noncoding RNA molecules, around 22 nucleotides long which, in mammals, are involved in repressing messenger RNA (mRNA) expression [89].

In mammals, miRNAs are normally generated from primary transcripts (pri-miRNAs) produced by RNA polymerase II, but some may be produced by RNA polymerase III. Within the primary transcript, the miRNA-encoding portions form imperfect stem-loop hairpin structures. Then, the Drosha type III RNase, the double-stranded RNA-binding protein DiGeorge syndrome critical region (DGCR8), together with other proteins, form a nuclear protein complex called "Microprocessor", which cleaves the hairpin loop pre-miRNAs away from the primary transcript. This allows the export of these pre-miRNAs into the cytoplasm. Here, variant forms of a canonical miRNA, called isomiRs, can be formed as an alternative cleavage by Drosha [90]. There, they are further processed by another protein complex containing the Dicer type III RNase; the miRNA is thus cleaved to its mature size. Once more, Drosha can induce an alternative cleavage leading to the formation of isomiRs. Finally, the mature strand of the miRNA duplex becomes associated with a final protein complex, called microRNA-induced silencing complex (miRISC), while the other strand is degraded. This complex contains proteins of the Argonaute (AGO) family, and its function is the silencing of genes targeted by the miRNA through mRNA recognition, typically at their 3' untranslated regions (3' UTR) [91, 92]. This mechanism is summarized in Figure 1.3.

There are two ways through which miRNAs repress mRNA. The first mechanism is the most common in plants. When there is a high degree of complementarity between the miR and the target mRNA, the AGO proteins of the miRISC complex promote the catalytic cleavage of the transcript, inducing its degradation. In mammals, however, the most typical mode of action does not involve target transcript cleavage and degradation; rather, silencing can be induced by the miRISC complex via deadenylation, translational repression, decapping and 5'-3' degradation. The AGO protein of the miRISC complex recruits several other proteins, including the ones from the GW182 family. Together, they mediate the interaction between AGO and downstream effector complexes, such as cytoplasmic deadenylase complexes, and the cytoplasmic poly(A)-binding protein (PABPC). Particularly, they promote the deadenylation of the poly(A) tail of the mRNA, which prompts decapping and 5'-3' degradation, and thus destabilization of the transcript. At the same time, the proteins recruited by miRISC are able to inhibit translation initiation

of the mRNA target of the miR. The mechanism is not completely known, but it is hypothesized that it happens through miRISC interacting with the eukaryotic initiation factor 4F complex (eIF4F) [93].

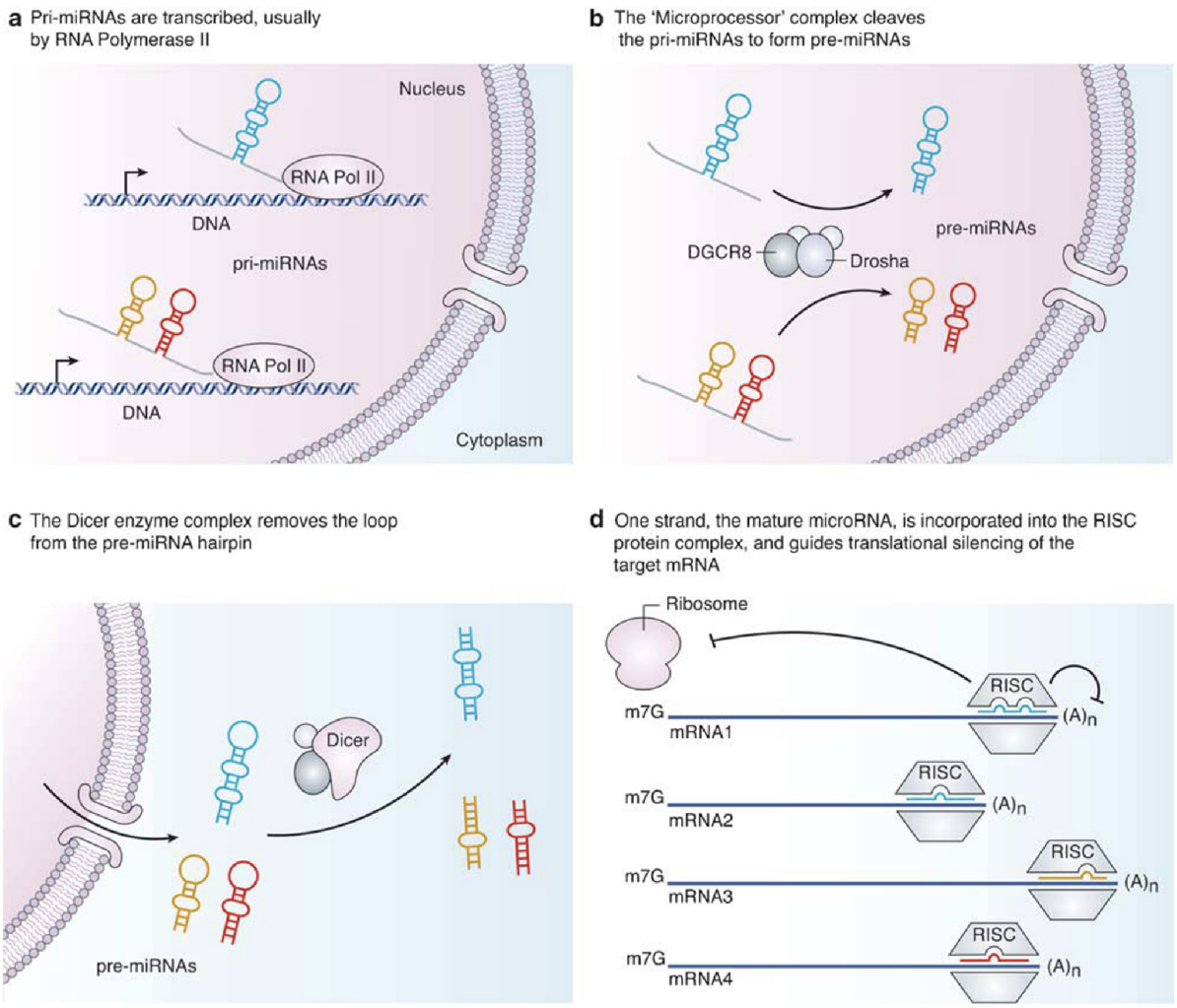


Figure 1.3: MicroRNA synthesis and function. **(a)** Pri-miRNAs are transcribed, usually by RNA Polymerase II. **(b)** The 'Microprocessor' complex cleaves the pri-miRNA to form the pre-miRNAs. **(c)** The Dcier enzime complex removes the loop from the pre-miRNA hairpin. **(d)** One strand, the mature microRNA, is incorporated into the RISC protein complex, and guides translational silencing of the target mRNA. From [91].

1.3.1 MicroRNAs in T cells

MicroRNAs have been widely studied in T cells, and they can either promote or suppress their activation, differentiation and/or function [88]. Supporting this is the fact that the microRNA landscape dramatically changes upon T cell activation [94]. Furthermore, cells deficient in components of the miRNA-machinery (e.g. Drosha or Dicer) exhibit impaired T cell proliferation and survival upon *in vitro* stimulation, and abnormal T cell population balance, with increased IFN-γ production by Th cells, for example [95, 96].

The disrupted balance between T cell subsets leads to unbalanced pro-inflammatory cytokine production which may lead to exacerbated inflammation or autoimmunity [88,97], as briefly indicated for subsets producing either IFN-γ or IL-17 in the previous subsection (see subsection 1.2.2) [43–46]. For the purpose of this Thesis, the following paragraphs will focus mainly on miRNA-mediated regulation of the pro-inflammatory αβ T cell counterparts of γδ T cells — Th1 and Th17 cells —, on miRNA-mediated regulation of γδ T cell differentiation, and then specifically on the current knowledge of miR-181a-mediated regulation of the development and differentiation of both αβ and γδ T cells.

1.3.1.A MicroRNAs in Th1 cells

As aforementioned, Th1 cells are $CD4^+$ cells whose signature cytokine is IFN-γ (see subsection 1.2.2). These cells play a role in the defense against extracellular pathogens and cancer, but oversecretion of IFN-γ may lead to autoimmunity [8].

T cells with genetic impairment of Drosha, DGCR8 or Dicer show increased T-bet and IFN-γ expression, and decreased Th1 cell numbers are also observed [88, 95, 96].

miRNAs can promote or suppress differentiation, either directly, by acting on key factors directly required for their differentiation, or indirectly, by regulating modulators of those factors. One example of a miRNA that promotes Th1 differentiation is miR-155, with miR-$155^{-/-}$ mice exhibiting higher resistance to EAE when compared to WT littermate controls [98]. Activated $CD4^+$ cells downregulate the IFN-γRα chain, thus activating miR-155 expression [99]. It then inhibits SHIP1 [98], which is a negative regulator of the phosphoinositide 3-kinase (PI3K) pathway [100], a signaling pathway with a major role in $CD4^+$ T cell differentiation [101]. It is thus possible that this miRNA is downregulating an IFN-γ receptor responsible for the differentiation of $CD4^+$ T cells into Th1 cells [88, 99].

Another example of a promotor of Th1 differentiation is miR-17-92 cluster [102]. The main player is miR-19b, who acts via Pten, which is also a negative regulator of the PI3K pathway, leading to Th1 differentiation and IFN-γ production [103]. A recent paper implicated miR-92 as having a role in neuroinflammatory responses in EAE by affecting Th1 differentiation possibly by downregulating TSC1 and DUSP10 [104].

On the other hand, miR-29 is an example of a microRNA that represses Th1 differentiation by directly targeting the TFs T-bet and Eomes [105], and the IFN-γ mRNA as well [106]. Recently, it has been postulated that Notch1 could be responsible for the priming of $CD4^+$ T cell responsiveness to Th1 polarization through the repression of the miR-29 family [107]. By directly targeting the IFN-γ "pathway", miR-29 suppresses Th1 differentiation, which is highly important in cell-mediated immunity, having direct implications in various conditions, like allergic rhinitis, bacterial infection, or even multiple sclerosis (MS) [108–110]. In the case of MS, patients exhibit an increased frequency of Th1 cells, accompanied by an increase in miR-29 expression as well [106].

1.3.1.B MicroRNAs in Th17 cells

Th17 cells are $CD4^+$ cells whose signature cytokines are IL-17A, IL-17F, IL-21 and IL-22 (see subsection 1.2.2). These cells play a role in the defense against fungi and extracellular bacteria, but they also contribute to increased severeness of inflammatory diseases like psoriasis, MS in humans and EAE in mice [8]. Drosha-deficient mice have higher numbers of Th17 cells [88, 95, 96].

A miRNA known to promote Th17 differentiation is miR-326, whose upregulated expression correlates with increased severity of EAE in mice and MS in humans. This miRNA acts by repressing Ets-1, a negative regulator of Th17 differentiation [111]. A recent study found that increased Th17 frequency in peripheral blood mononuclear cells (PBMCs) associated with miR-326 is linked to Hashimoto's thyroiditis in humans, possibly by targeting IL-23 and IL-23R [112]. miR-326 causes Th17 polarization and increases methylation of FOXP3, which promotes the inflammatory state in the adipose tissue of obese individuals [113].

miRNA-384 also promotes Th17 differentiation i*n vitro*, with its expression being linked to EAE severity *in vivo*. By dual luciferase reporter assay, SOCS3 was identified as a direct target of this miRNA [114]. Interestingly, a recent study has shown that STAT3 regulates miR-384 during Th17 differentiation [115].

A novel study with miR-155 identified hundreds of cellular mRNAs targets that were cell-specific, indicating a cell-context-dependent miR-mediated regulation of gene expression [116]. This miRNA was associated to having a detrimental role in dextran sulfate sodium (DSS)-induced colitis in intestinal mucosal cells of mice by targeting Jarid2 and inducing Th17 differentiation [117]. Accordingly, a miR-155 antagomir — engineered oligonucleotide that binds to the mRNA target site of the miRNA — ameliorated DSS-induced colitis symptoms through the Jarid2/Wnt/β-catenin axis [118]. Higher miR-155 expression has been correlated to severe pancreatitis, and SOCS1 has been identified as its target [119].

Conversely, the miR-106-363 cluster is associated with Th17 suppression. Transfection of miR-18b, miR-106a and miR-363-3p into primary murine $CD4^+$ lymphocytes *in vitro* led to decreased expression of *Rorc*, *Rora*, *Il17a* and *Il17f*, and halted IL-17 secretion. Using luciferase reporter assays, RORα and NFAT were confirmed as targets [120]. Th17 inhibition has been reported individually by members of this cluster. For instance, miR-106a deficiency associated with decreased Th17 frequency leads to attenuated inflammation of an inflammatory bowel syndrome (IBS) murine model [121].

A recent paper describes an interesting mechanism between miR-122 and miR-123 and intestinal Th17 cell response by using global knockout (KO) mice for both miRNAs [122]. miRs-221/222 were shown to be necessary to contain IL-17 production upon activation. By scRNA-seq, the healthy mice were discovered to maintain Th17 intestinal balance in the absence of these microRNAs. Also, a bioinformatical analysis identified *MAF* and *Ilr23* to be targets of these miRNAs. Using the DSS colitis model, miR-221/222-KO mice had increased symptom severity when compare to the WT mice, which was accompanied by an increase in Th17 cells in a T cell specific fashion. In conclusion, miRs-221/222

were shown to act as important negative feedback regulators downstream of IL-23 and to be essential components of intestinal Th17 cells to contain pro-inflammatory responses.

1.3.1.C MicroRNAs in γδ T cells

In mice conditionally lacking Dicer, one observes an increase in γδ T cells in the DN thymic compartment [15, 123]. However, there are very few studies exploring microRNAs in γδ T cell differentiation.

miR-133b and miR-206, who are directly upstream of the *Il17a/f* gene locus, were shown to be highly expressed in *in vitro* polarized Th17 cells and γδ17 T cells, with IL-23 being crucial for their expression [124].

Schmolka et al. studied the role of miR-146a in γδ T cell plasticity [125]. miR-146a was first identified as being overexpressed in γδ27$^-$ CCR6$^+$ of peripheral lymphoid murine cells, a pattern that was also observed in thymocytes, prompting the conclusion that this miRNA is already differentially expressed during γδ T cell development, a signature that is maintained in the periphery. Overexpression of this miR in fully differentiated γδ T cells from the spleen and lymph nodes has led to a notable reduction in IFN-γ$^+$ γδ T cells, suggesting that miR-146a is inhibiting IFN-γ production. Using γδ T cells isolated from miR-146a-KO mice, it was shown that *in vitro* stimulation with IL-1β and IL-23 resulted in a significant increase in double producer frequency in KO cells when compared to WT γδ T cells. After *Listeria monocytogenes* infection, miR-146a$^{-/-}$ mice presented more double producing γδ T cells cells than WT littermate controls. A subsequent differential Ago2 RNA immunoprecipitation followed by sequencing (Ago2 RIP-seq), allowed for the unbiased identification of *Atf2* and *Nod1* as potential miR-146a targets, which were further validated by luciferase reporter assays. However, the expression pattern between γδ27$^+$ and γδ27$^-$ CCR6$^+$ cells was only significant for *Nod1*, being significantly higher expressed in γδ27$^+$ cells. Finally, γδ27$^-$ CCR6$^+$ cells Nod1$^{-/-}$ mice failed to differentiate into double producers, and they were increasingly susceptible to *L. monocytogenes* infection *in vivo*. It was thus concluded that the miR-146a/NOD1 axis is crucial for γδ T cell plasticity and differentiation.

miR-17 was linked to γδ T cell development in humans, mediated by a Notch-driven negative feedback loop [126]; however, no effect is described for this miRNA on pro-inflammatory subset production.

Recently, the miRNA expression profiles of peripheral γδ and αβ T human cells were characterized, leading to the identification of 14 differentially expressed miRNAs [127]. Of these, miR-125b-5p and miR-99a-5p were downregulated in activated T cells, with functional overexpresion studies showing induced apoptosis and inhibited γδ T cell activation. Furthermore, inhibitors of these miRs enhanced cytotoxicity to tumor cells, and miR-125b-5p inhibition and co-culture with tumor cells for 48 hours significantly increased IFN-γ and TNF-α secretion.

1.3.2 miR-181

The miR-181 family is a highly conserved family of microRNAs in vertebrates [128]. For both human and mice, it consists of four members: miR-181a, miR-181b, miR-181c and miR-181d. It is preferentially expressed in several tissues, like in the bone marrow, spleen and thymus, in the retina, and in the brain [129–131], and it has been implicated in several biological processes, ranging from myoblast differentiation to various types of cancer [132, 133].

1.3.2.A miR-181 in T cells

miR-181 and, in particular, isoform miR-181a, is highly expressed at the DP stage of thymocyte development [134], and it has been described as an important regulator of T cell sensitivity to antigens and in promoting T cell activation [135, 136].

In the seminal work by Li et al., the authors disclosed the effect of miR-181a as a rheostat of TCR sensitivity [135]. They assessed miR-181a expression in different steps of thymocyte development, and found that is was dynamically regulated during T cell maturation — it was high from stages DN1-3 and particularly in DP, but that it dropped in DN4, and ISP $CD4^+$ and ISP $CD8^+$. In fact, in another paper by the same authors, and using an OP9-DL1 stromal cell and thymocyte co-culture assay, which recapitulates early thymic T cell development in culture, they showed that miR-181a overexpression increases the amount of DP cells, while decreasing the amount of $CD8^+$ SP cells [137]. Subsequent measurements of calcium levels were performed in primed T cells overexpressing miR-181a in order to assess TCR signaling strength. Indeed, miR-181a overexpression led to a significant increase of intracellular calcium, indicating that its expression amplifies TCR-mediated T cell activation. Mechanistically, such changes were not the result from changes in the expression of surface receptors, but rather through the repressed expression of multiple inhibitory phosphatases; these include the tyrosine phosphatases: SHP-2 and PTPN22, and also DUSP-5 and -6 from the ERK pathway [138]. These phosphatases have been shown to inhibit TCR signaling by phosphorylation of members of the ERK pathway, or by dephosphorylation of Lck, Zap70 or CD3-associated ITAMs [60, 139]. Antagomir repression of miR-181a expression levels leads to impairment of thymocyte positive selection [135]. One other study showed that miR-181a is in part responsible for setting a TCR signalling threshold in developing thymocytes, preventing overreaction to antigens during positive selection [136].

Ablation of miR-181a leads to dampening of TCR and ERK signaling, thus increasing the threshold of positive selection, but this does not cause an autoimmune phenotype [140]. Interestingly, additional studies in humans link the decline of miR-181a expression with age with impaired TCR sensitivity by increased DUSP6 activity in $CD4^+$ T cells [141], which is concomitant with a decline in expression of the TF YY1 [142]. PTPN22 has been found to react to TCR signaling in naive T cells by limiting TCR signaling by weak agonists and self-antigens, while permitting responses to strong agonists [143]. One

paper reports miR-181a to be responsible for sustained ERK phosphorylation in Th17 cells, which leads to them having a lower threshold of activation to antigens when compared to other Th cells; *Id3* was pinpointed to be activated upon this sustained phosphorylation and to negatively regulate *Rorc* and, consequently, *Il17* [144].

miR-181a has been discovered to be a negative regulator of IFN-γ expression in $CD8^+$ cells, players of the adaptive immune system that fight intracellular infections and cancer, and which, upon activation and differentiation into cytotoxic T lymphocytes (CTLs), secrete IFN-γ as their main cytokine [145]. In particular, it limits IFN-γ production by suppressing *Id2*, which then dampens *Ifng* expression. miR-181a-deficient mice also generate more IFN-γ-producing $CD8^+$ T cells after viral infection, making those mice more effective in controlling the viral infection.

Interestingly, miR-181a/b has decreased expression levels in MS patients and EAE mice. Its expression was found to inhibit Th1 differentiation and to promote regulatory T cell (Treg) differentiation in $CD4^+$ T cells, with Smad7 being directly suppressed by this miRNA, making it an important regulator of the T cell response in a context of neuroinflammation [146]. Tregs are a subpopulation of $CD4^+$ T cells that work alongside Th1 and Th17 cells to maintain homeostatic balance by limiting effector T cell responses and containing inflammation [8, 88]. miR-181a/b expression has been found to control Treg development intrathymically by acting on CTLA-4, a mechanism became imprinted after their migration to the periphery [147]. One study reports that miR-181a enhances TCR signal strength, which increases NFAT5 expression levels, leading to impaired Treg expansion and increased autoimmunity [148].

NKTs are innate-like T lymphocytes from the thymus that recognize glycolipid antigens presented by the MHC-I-related glycoprotein CD1d [149]. Invariant NKTs (iNKT) are the major subset of NKTs, who have a very restricted TCR repertoire and express Vα14-Jα18 and Vβ8.2, -7 or -2 chains in mice [150]. One of their main characteristics is the capacity to rapidly provide a set of pro-inflammatory cytokines (e.g. IFN-γ and IL-4) upon TCR engagement (i.e. upon encounter with pathogens) [151]. An interesting mechanism regarding miR-181a/b1 and iNKTs has been described, in which abrogation of these miRNAs led to a dramatic reduction of iNKT numbers in early thymocyte development, a phenomenon that was imprinted into the periphery [152]. Moreover, DP thymocytes from miR-181a/b1-deficient mice had impaired signaling after TCR stimulation and lower cytokine production in the periphery. miRNA expression rescuing via agonists restores iNKT cell development in KO mice.

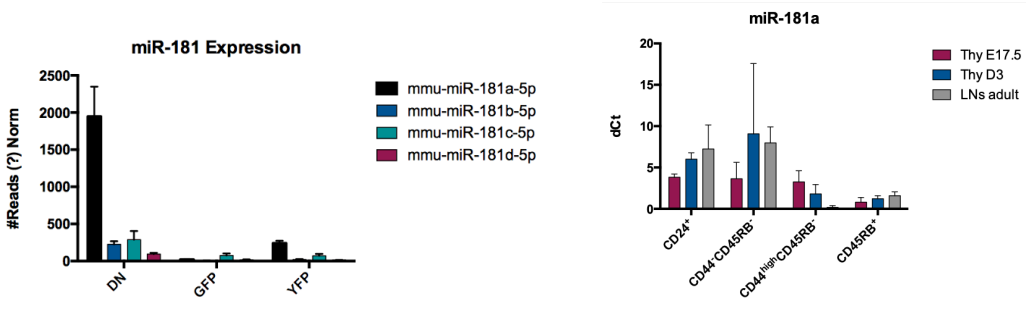
Finally, one paper reports that the miR-181a/b-1 cluster does not affect γδ T cell cell numbers in the thymus nor in the lymph nodes, and that it has no effect in steering naive cells towards an IFN-γ or IL-17 fate [153]. However, preliminary data obtained in the host lab in γδ T cell subsets isolated from a double reporter mouse model for IFN-γ and IL-17 allowed for a more detailed characterisation of miR-181a expression levels, which will be explored in this study.

1.4 Preliminary Results

The host lab performed preliminary studies to determine whether miR-181 was differentially expressed in γδ T cell subpopulations. To achieve that, double reporter mice were used, where IFN-γ was co-translated with eYFP$^+$ and IL-17A was co-translated with GFP$^+$, thus allowing for the isolation of both IFN-γ$^+$ and IL-17A$^+$ γδ T cell subpopulations.

Peripheral T lymphocytes of adult mice were isolated from the lymph nodes and the IL-17A$^-$GFP$^-$IFN-γ$^-$eYFP$^-$ (DN), IL-17A$^+$ and IFN-γ$^+$ subsets of γδ T cells were sorted and subjected to small RNA-seq. This analysis revealed 103 differentially expressed miRNAs between the two γδ T cell effector populations, with miR-181a being slightly overexpressed in IFN-γ$^+$eYFP$^+$ cells compared to IL-17A$^+$GFP$^+$ cells, and mostly overexpressed in the double negative DN subset, when compared to the effector subsets. The remaining isoforms — miR-181b, miR-181c, miR-181d — did not show any significant differences within the γδ T cell subsets analysed (see Figure 1.4(a)).

Importantly, the expression of miR-181a in cells from the E17.5 thymus, from the D3 thymus and from the peripheral lymph nodes (pLNs) of adult mice was further characterized by RT-qPCR in γδ T cell populations based on the expression of the CD24, CD44 and CD45RB cell surface markers (see Figure 1.4(b)). Of note, CD24 expression characterizes immature γδ T cells, the CD44^{hi}CD45RB$^-$ subset represents the IL-17-associated subset of γδ T cells, and CD45RB expression characterizes the IFN-γ subset of γδ T cells. In this analysis, the relative expression of miR-181a was the most prominent in the immature (CD24$^+$) and in the uncommitted subset (CD44$^-$CD45RB$^-$) of the neonatal thymus, and of the pLNs of adults. These differences in expression levels are the basis of the studies performed in this Thesis, as detailed in the objectives defined in the next section.



(a) **(b)**

Figure 1.4: Preliminary studies of miR-181 expression. **(a)** Next generation sequencing results of the expression of each isoform of miR-181 in the IL-17A$^-$IFN-γ$^-$ (DN), IL-17A$^+$ and IFN-γ$^+$ subsets of γδ T cells isolated from peripheral T lymphocytes of adult mice. **(b)** miR-181a expression in the CD24$^+$, CD44$^-$CD45RB$^-$, CD44^{hi}CD45RB$^-$ and CD45RB$^+$ subsets from the E17.5 thymus, the D3 thymus and the peripheral lymph nodes of adult mice.

2

Objectives

MicroRNA-181a has been shown to be a regulator of TCR sensitivity to antigens and to promote T cell activation [135]. However, its specific role in γδ T cell function is not clear. Indeed, a previous study showed that miR-181a does not impact γδ T cell numbers neither in the thymus, nor the periphery [153], but results from the host lab show high expression levels of miR-181a in specific γδ T cell subsets throughout development (see Section 1.4), making it a potential modulator of γδ T cell effector commitment, possibly by affecting TCR signaling strength. To test this hypothesis, this study will assess the role of miR-181a in the development and differentiation of the pro-inflammatory subsets of γδ T cells by using a loss-of-function approach based on the functional characterization of miR-181a-deficient mice. As such, we will:

1. Determine the role of miR-181a in γδ T cell development by analyzing fetal, neonatal, and adult γδ T cell subpopulations isolated from *ex vivo* lymphoid organs of either miR-181a-deficient or WT mice;
2. Assess the signaling capacity of miR-181a-deficient and WT γδ T cells by measuring the levels of calcium that are mobilised into the cytosol;
3. Study the impact of miR-181a on TCR signaling during γδ T cell differentiation *in vitro*;
4. Identify potential miR-181a targets in γδ T cells by bioinformatical analysis.

3

Materials and Methods

Contents

3.1 Mice

We used miR-181a/b-1 KO mice (B6.*Mirc14*$^{tm1.1Ankr}$), which were described in [152]. All studies were performed using both miR-181a-KO mice and WT littermate controls. All mice were studied at the fetal (E17.5), neonatal (3 days), or adult (8–12 weeks) stages.

Mice were bred and maintained in the specific pathogen–free animal facilities of Instituto de Medicina Molecular João Lobo Antunes (Lisbon, Portugal). All experiments involving animals were done in compliance with the relevant laws and institutional guidelines and were approved by the ethics committee of Centro Académico de Medicina de Lisboa.

3.1.1 Timed matings

Male and female adult mice with specific genotypes were selected to undergo a timed mating protocol in order to obtain mice with the age required for each analysis. Genotyping of the new generations was performed on a small piece of ear or tail.

48h prior to induction, 2 female mice were placed inside an empty cage that a male mice had previously occupied, whereas the male was placed in a new cage. The purpose of this was to align the hormonal cycles of the females in such a way that the chance of a pregnancy occurring was optimal. On the day of the induction, the females were transferred to the cage of the male and left to breed overnight, after which females and male were separated and the females checked for plugs, which indicated whether the mice had copulated and indicating possible pregnancies. The gestation period is around 21 days, after which mice were born and genotyped, except for the experiments with fetal mice.

3.1.2 Genotyping

We performed DNA extraction from a piece of the tail of each mice using the Xpert directXtract PCR kit (GRiSP)), which was followed by PCR using the Xpert Fast DNA Polymerase. The cycling conditions were as indicated: (i) 95 ℃ for 3 min, (ii) 95 ℃ for 15 s, (iii) 60 ℃ for 15 s, (iv) 72 ℃ for 2 s, and (v) 72 ℃ for 2 min. Steps (ii) to (iv) were repeated 40 times. To genotype miR-181a/b1 KO animals, the following primers were used:

1. forward primer 5′-TCAGCACATCTCTGCCTCAC-3′,
2. reverse primer 5′-AGGGAACGTCTGTTGATTGC-3′.

The size of PCR products of knockout and WT alleles are 150 base-pairs (bp) and 300 bp, respectively (see Figure 3.1). The PCR reactions were done using MyCycler Thermal Cycler (BioRad).

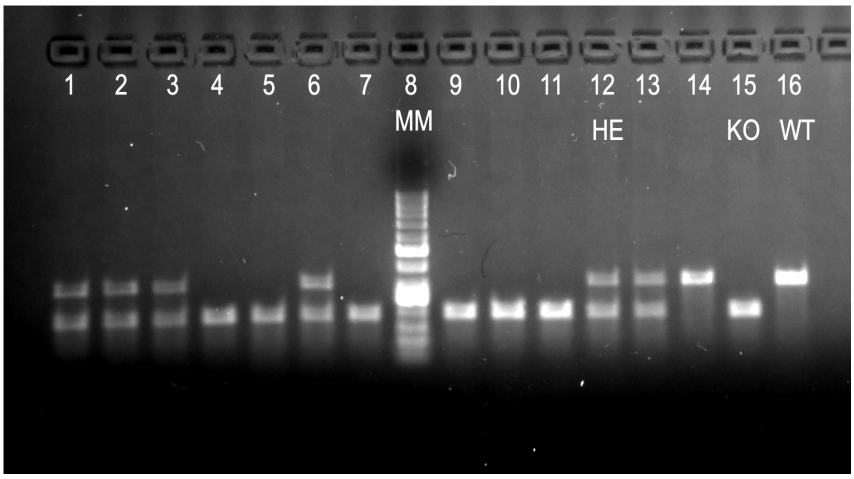


Figure 3.1: Agarose gel with genotyping results from miR-181a/b1-KO mice. The size of PCR products are 150 bp for KO mice (only bottom band present), and 300 bp for WT mice (only top band present); columns with both bands belong to heterozygous mice.

3.1.3 Dissection

The peripheral lymph nodes (cervical, axillary, inguinal, and popliteal), and spleen were dissected from adult mice, and the thymus was dissected from fetal and neonatal mice. Until the cell preparation phase, organs were kept at 4 ℃ and in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% HEPES buffer, 1% nonessential amino acids (NEAA), 1% sodium pyruvate, 1% penicillin and steptomycin, 0.1% gentamicin and 0.1% β-mercaptoethanol.

3.2 Cell Preparation

Cell suspensions were obtained from thymus, spleen or peripheral lymph nodes. Erythrocytes were osmotically lysed in red blood cell lysis buffer (BioLegend). Cells were filtered through 70-µm cell strainers (BD Biosciences). Cell suspensions were washed and homogenized using 1x phosphate buffered saline (PBS).

3.3 Monoclonal Antibodies

The following anti-mouse fluorescently labeled monoclonal antibodies (mAbs), antigens and clones, were used: TCRδ (GL3), TCRβ (H57-597), Vγ5 (536), Vγ1 (2.11), Vγ4 (UC3-10A6), IgG (Poly4055), CD3 (145.2C11), CD4 (GK1.5), CD8 (53–6.7), CD24 (M1/69), CD27 (LG.7F9), CD44 (IM7), CD45 (30-F11), CD45RB (C363-16A), Ki-67 (16A8), IFN-γ (XMG1.2), and IL-17A (TC11.18H10.1). Antibodies were purchased from BD Biosciences, eBiosciences, or BioLegend.

3.4 Flow Cytometry and Cell Sorting

For cell surface staining, single-cell suspensions were incubated for 10-30 minutes with saturating concentrations of mAbs. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (1 µg/mL) in the presence of brefeldin A (1 µg/mL) (all from Sigma-Aldrich) for 3 hours at 37 ℃. Cells were stained for the aforementioned cell surface markers in the presence of anti-CD16/CD32 (eBioscience). For intracellular staining, cells were additionally fixed for 30 minutes at 4 ℃, permeabilized with the Foxp3/Transcription Factor Staining Buffer set (eBioscience) in the presence of anti-CD16/CD32 (eBioscience) for 15 minutes at 4 ℃, and lastly incubated for 30-60 minutes at 4 ℃ with the antibodies against intracellular proteins in permeabilization buffer. The cells were sorted on FACSAria (BD Biosciences) or analyzed using FACSFortessa (BD Biosciences). Data was analyzed using FlowJo software (Tree Star).

3.5 Calcium Flux Assay

Cell suspensions were incubated for 1 hour at 37 ℃ with a Ca^{2+} die by using the PBX Calcium Assay kit (BD), consisting of the calcium indicator PBX, a 10 x PBX signal enhancer and a calcium assay buffer. Cells were then stained for the aforementioned cell surface markers for 15 minutes at 4 ℃, washed and kept at 4 ℃. Samples were pre-incubated at 37 ℃ for 10 minutes prior to the assay, upon which they were placed in the flow cytometer and baseline data was acquired for 40 seconds, after which 8 µL if anti-CD3 (0.5 mg/mL) was added, data was acquired for another 40 seconds, and, finally, 80 µL of anti-IgG (0.5 mg/mL) was added and data was acquired for $\sim$ 8 minutes, all using FACSFortessa (BD Biosciences).

3.6 *In Vitro* Stimulation of the γδ27$^-$ and γδ27$^+$ Subsets

γδ27$^+$ and γδ27$^-$ cells were isolated from the pLNs of adult mice, and sorted using fluorescence-activated cell sorting (FACS), and cultured overnight at 37 ℃ in the presence of plate-bound anti-CD3ε (145.2C11) plus anti-CD28 mAb (37.51), both at 2.5 µg/mL (PeproTech). This protocol allowed for short-term TCR stimulation.

3.7 Target Gene Prediction

Predicted and validated targets for mmu-miR-181a were determined using miRWalk 3.0 [154]. This database uses a machine learning approach to find predicted and validated miRNA-target interactions

in up-to-date databases. To predict novel targets, it uses the TarPmiR algorithm [155], and theTargetscan and the miRDB platforms [156,157]. The validated targets included in miRWalk 3.0 are from miRTarBase [158].

3.8 Statistical Analysis

The statistical significance of differences between two populations was assessed using either the two-tailed nonparametric Mann-Whitney test or the t-test when applicable. ANOVA, followed by multiple comparisons, was performed when comparing the mean of more than one population. P values $\leqslant 0.05$ were considered significant and are indicated in the figures. In bar graphs, data is presented as mean $\pm$ SD.

4

Results

Contents

As mentioned in the first chapter (see Section 1.4), sequencing results from the host lab have shown that miR-181a is highly present in the DN subset (IFN-γ^-IL-17^-) of adult peripheral γδ T cells, when compared to IFN-γ^+ or IL-$17A^+$ subsets. Also, miR-181a was shown to be particularly overexpressed in immature $CD44^-CD45RB^-$ cells of adult and neonatal mice, suggesting a functional role for miR-181a in γδ T cell development. These data, along with information from the literature (see Section 1.3.2), motivated further studies, aiming at characterizing the role of miR-181a in γδ T cell biology. As such, in this work, we did several experiments comparing miR-181a-KO and WT littermate control mice under different conditions. We started by performing *ex vivo* analyses of γδ T cell populations isolated from lymphoid organs at different developmental stages. We first analyzed γδ T cells from the peripheral lymph nodes and spleen of adult mice, which was followed by the characterisation of γδ T cells from the thymus of fetal (E17.5) and neonatal (D3) mice. To determine a potential role for miR-181a in TCR signaling of γδ T cells, we performed calcium flux assays using γδ T cells isolated from the peripheral lymph nodes of adult mice. To infer the potential of miR-181a to regulate γδ T cell differentiation, we did a short *in vitro* stimulation of the γδ27^- and γδ27^+ subsets from the pLNs of adult KO and WT mice and determined IFN-γ and IL-17 expression levels. Finally, to better understand the biological role of miR-181a in regulating γδ T cell differentiation and function, we did a bioinformatical search to identify potential targets of miR-181a in γδ T cells.

4.1 *Ex Vivo* Analysis of γδ T Cells from Lymphoid Organs of WT and miR-$181a^{-/-}$ mice

We assessed several parameters of γδ T cells isolated from lymphoid organs *ex vivo* through Flow Cytometry (see Figure 4.1), and at different developmental stages. These analyses were performed in adult miR-$181a^{+/+}$ and miR-$181a^{-/-}$ mice of 8 – 12 weeks of age, in mice at the embryonic day E17.5 and in neonatal D3 pups.

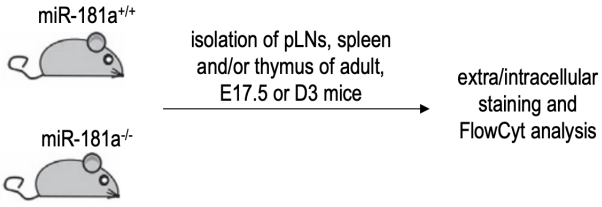


Figure 4.1: *Ex vivo* analysis of cells isolated from the pLNs, spleen and/or thymus of miR-$181a^{+/+}$ and miR-$181a^{-/-}$ mice. Isolated cells were processed and subjected to extra- and intracellular staining, and analysed by Flow Cytometry.

4.1.1 *Ex vivo* analysis of adult lymphoid organs

The preliminary data highlighted miR-181a overexpression in the uncommitted CD44$^-$CD45RB$^-$ γδ T cell subset of adult mice (see Figure 1.4(b)). These observations led us to hypothesise that miR-181a might play a role in γδ T cell development/differentiation. To test this, we first investigated the overall lymphocyte behavior and the specific γδ T cell patterns of *ex vivo* cells isolated from adult lymphoid organs of miR-181a$^{-/-}$ mice and their WT littermate controls. Based on the miR-181a expression profile, we expected to see the most pressing disparities within the CD44$^-$CD45RB$^-$ subset. Specifically, we isolated cells from the pLNs and spleen of adult mice (8 – 12 weeks), did extra- and intracellular fluorescent staining of the isolated cells, and analyzed the data using flow cytometry, as indicated in Figure 4.1.

Firstly, for both organs, there was no difference in terms of frequency or total cell number of the Live CD45$^+$ population between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice (see Figure 4.2(a)), indicating that miR-181a does not affect overall lymphocyte cell survival. Of note, CD45 expression allows for the identification of cells belonging to the HSC lineage [159]. Concerning γδ T cells, their frequency was significantly higher in miR-181a$^{-/-}$ mice when compared to the WT littermate controls, but cell surface staining of KI-67 on γδ T cells was similar between WT and KO animals (see Figure 4.2). This indicates that miR-181a might somehow influence γδ T cell production, without impacting γδ T cell proliferation in adult mice.

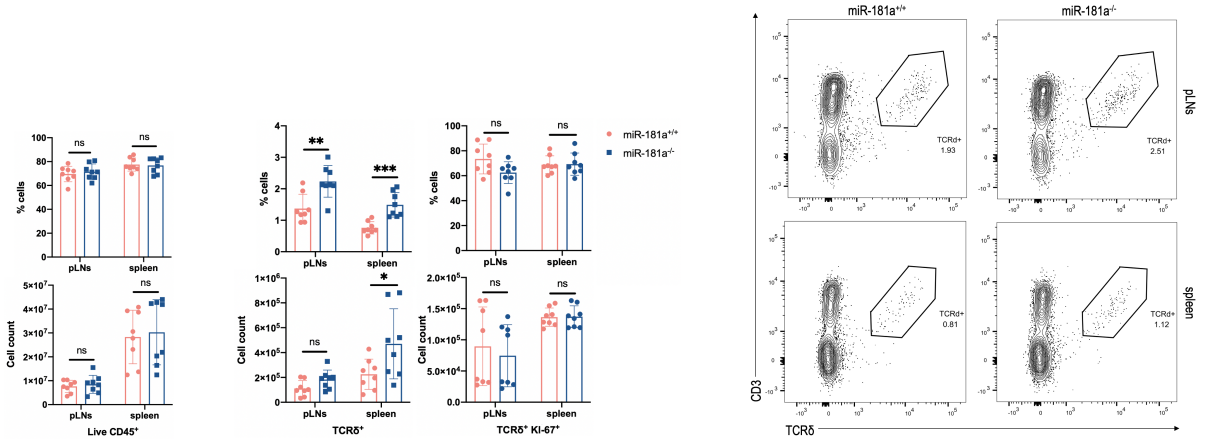


Figure 4.2: Comparison of the frequency and number of Live CD45$^+$ cells, overall γδ T cells, and KI-67$^+$ γδ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ lymphoid organs of adult mice. **(a)** Frequency and total cell number of Live CD45$^+$ cells isolated from the pLNs and spleen of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice. **(b)** Frequency and total cell number of γδ T cells and KI-67$^+$ γδ T cells (Live$^+$CD45$^+$CD3$^+$TCRδ$^+$) isolated from the pLNs and spleen of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice. **(c)** Flow cytometry analysis of extracellular TCRδ expression in the pLNs (top) and spleen (bottom) between miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) adult mice. The data shown is from two independent experiments, each consisting of n = 4. Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01, *** $\leqslant$ 0.001 (Mann-Whitney two-tailed test).

A more detailed analysis of γδ T cells, showed that Vγ chain usage differed between WT and miR-181a-KO mice (see Figure 4.3), with the latter having a higher proportion of Vγ1- and a lower proportion of Vγ4-expressing γδ T cells, when compared to the former, and with the Vγ5 population being nearly absent in both groups, as expected, since the wave of production of Vγ5-expressing cells occurs between days E13 and E16, after which they populate the skin [35]. In the peripheral lymphoid organs of adults, Vγ1 expression is typically associated to IFN-γ provision, whereas Vγ4$^+$ cells can be both IL-17 and IFN-γ producers [47]. Since there is currently no commercially available antibody for Vγ6, its expression was inferred from the percentages of the remaining Vγ chains.

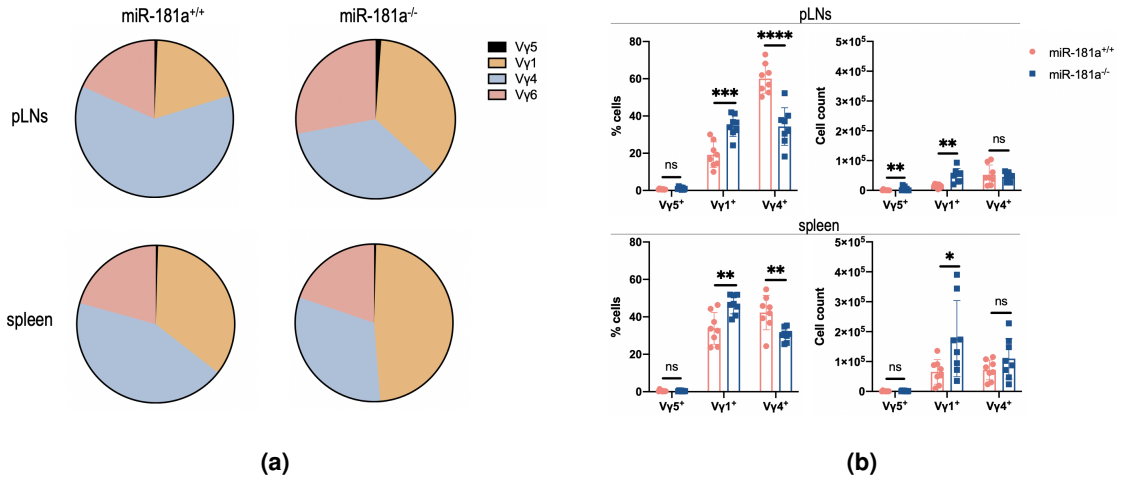


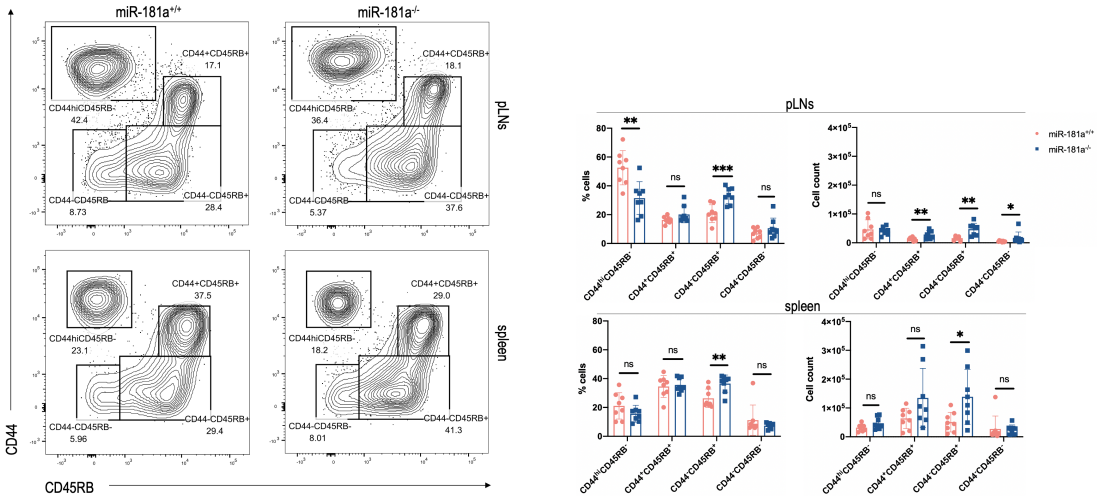
Figure 4.3: Comparison of Vγ chain usage by γδ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice, through extracellular fluorescent labelling of each Vγ chain and subsequent flow cytometry analysis. **(a)** Pie charts with the average proportion of Vγ chain expression, between miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ mice (right), for the pLNs (top) and the spleen (bottom). **(b)** Frequency and total cell number of Vγ5, Vγ1 and Vγ4 (CD3$^+$TCRδ$^+$CD24$^-$) usage within the pLNs (top) and the spleen (bottom), between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice. The data shown is from two independent experiments, each consisting of n = 4. Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01, *** $\leqslant$ 0.001, **** $\leqslant$ 0.0001 (Mann-Whitney two-tailed test).

To better understand the role of miR-181a in γδ T cell function, namely, on their potential of becoming effector cells, we compared γδ T cell subsets between WT and miR-181a-KO mice based on CD44 and CD45RB expression [57] (see Figure 4.4), in which CD44^{hi}CD45RB$^-$ correlates with IL-17 expression, and both CD44$^+$CD45RB$^+$ and CD44$^-$CD45RB$^+$ correlate with IFN-γ expression, with CD44$^+$ cells corresponding to more effector than the CD44$^-$ cells. Thus, and in agreement with Vγ expression, miR-181a$^{-/-}$ showed a decreased frequency of CD44^{hi}CD45RB$^-$, and an increased frequency and/or total cell count of CD44$^+$CD45RB$^+$ and CD44$^-$CD45RB$^+$, with the major difference being observed in the less effector population of the two. Despite CD44$^-$CD45RB$^-$ cells representing a minor population at this stage in development ($<$ 10%) [57], we had expected to observe a marked difference in this subset, since miR-181a was significantly overexpressed in this subset of adult mice (see Section 1.4); however, this population

was only slightly increased in KO mice, and that was only statistically significant in terms of total cell numbers in the pLNs.

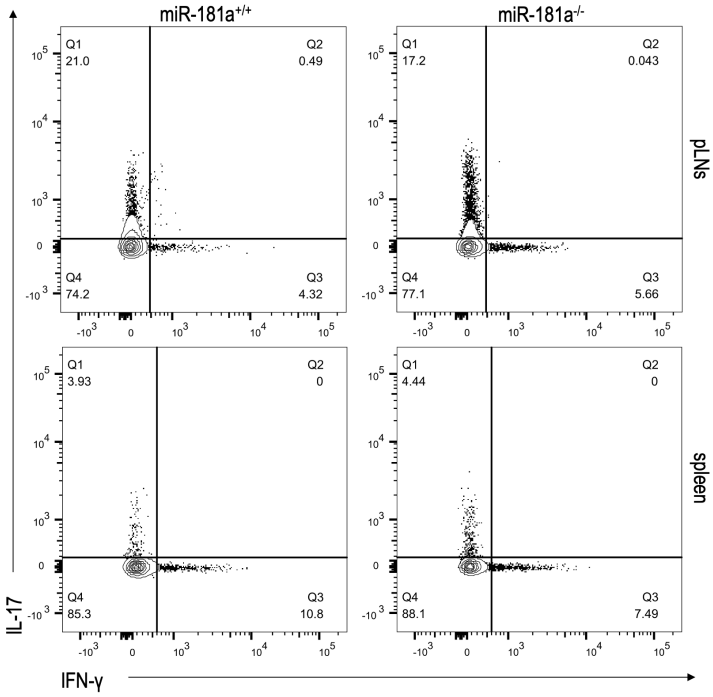
These differences did not translate into cytokine production by γδ T cells (see Figure 4.5), in that we could only observe a slight tendency for increased IFN-γ and decreased IL-17 expression, in terms of frequency and total cell numbers, in miR-181a$^{-/-}$ mice, and no difference could be observed in terms of Mean Fluorescence Intensity (MFI), prompting the conclusion that miR-181a does not affect cytokine production in the pLNs and spleen of adult mice in steady-state.

Considering these data, we thus hypothesized that miR-181a is likely to play a role in γδ T cell differentiation in adults, influencing not only the total number of γδ T cells, but also the proportion of its two pro-inflammatory subsets. Its presence is concomitant with lower γδ T cell frequency and numbers. Not only that, it can be correlated with increased Vγ4 and CD44^{hi}CD45RB^{-} expression — associated with the IL-17 subset —, and decreased Vγ1 and CD44^{+}CD45RB^{+} and CD44^{-}CD45RB^{+} expression — associated with the IFN-γ subset. To determine if these differences occur at an earlier stage of development in the thymus and become imprinted onto adulthood and into the periphery, we performed the same *ex vivo* analysis in fetal (E17.5) (see Section 4.1.2) and neonatal (D3) (see Section 4.1.3) thymic cells.

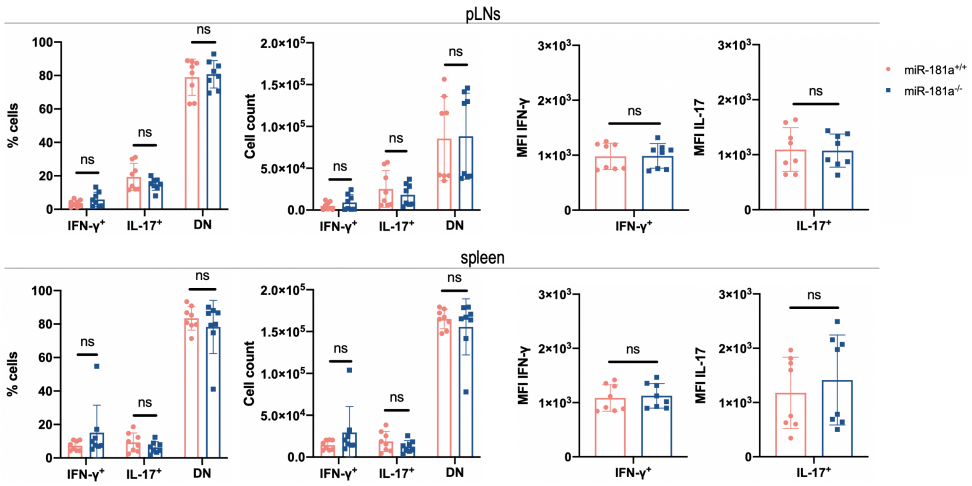


(a) **(b)**

Figure 4.4: Comparison of distinct γδ T cell subsets, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice, by extracellular fluorescent labelling of the CD44 and CD45RB cell surface markers and subsequent flow cytometry analysis. **(a)** Flow cytometry analysis of extracellular CD44 and CD45RB expression in the pLNs (top) and spleen (bottom), between miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) adult mice. **(b)** Frequency and total cell number of cells of four distinct γδ T cell subsets, CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+}, and CD44^{-}CD45RB^{-} (CD3^{+}TCRδ^{+}CD24^{-}), in the pLNs (top) and spleen (bottom), between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice. The data shown is from two independent experiments, each consisting of n = 4. Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01, *** $\leqslant$ 0.001 (Mann-Whitney two-tailed test).



(a)



(b)

Figure 4.5: Comparison of pro-inflammatory cytokine expression by γδ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice, by intracellular fluorescent labelling of IFN-γ and IL-17 and subsequent flow cytometry analysis. **(a)** Flow cytometry analysis of intracellular IFN-γ and IL-17 expression in the pLNs (top) and spleen (bottom) between miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) adult mice. **(b)** Frequency, total cell number of cells and/or MFI of DN, IFN-γ^{+} and IL-17^{+} cells ((CD3^{+}TCRδ^{+}CD24^{-}), in the pLNs (top) and spleen (bottom), between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice. The data shown is from two independent experiments, each consisting of n = 4. Each symbol represents an individual mouse. ns $P > 0.05$ (Mann-Whitney two-tailed test).

4.1.2 *Ex vivo* analysis of E17.5 thymus

After confirming that miR-181a affects the differentiation of pro-inflammatory γδ T cell subsets of adult mice, and given that these cells are capable of producing effector cytokines during embryonic development in the thymus (see Section 1.2.2), it became relevant to determine whether the differences observed in adult lymphoid organs were already imprinted earlier on in development. To investigate this possibility, we first performed *ex vivo* analysis of the fetal thymus of miR-181a$^{-/-}$ and miR-181a$^{+/+}$ mice. For that, we harvested the thymus of fetal mice (E17.5) and did extracellular staining for several cell surface markers and intracellular staining to detect IFN-γ and IL-17, followed by Flow Cytometry analysis (see Figure 4.1). Given the preliminary data obtained by the host lab, showing that miR-181a was not particularly overexpressed in γδ T cells in the E17.5 thymus when compared to other developmental stages (see Section 1.4), we did not expect to see any major differences in this case.

As with the adults, fetal mice showed no difference in terms of live cells between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice (see Figure 4.6(a)), indicating that miR-181a does not affect overall lymphocyte survival at this stage. Again, we observed an increased frequency of γδ T cells in miR-181a$^{-/-}$ mice when compared to miR-181a$^{+/+}$ mice, but that difference did not translate into absolute cell numbers (see Figure 4.6(b)). We did not see a difference in terms of KI-67^{+} γδ T cells. This indicates that miR-181a possibly plays a role in γδ T cell development in the fetal thymus, without affecting γδ T cell proliferation.

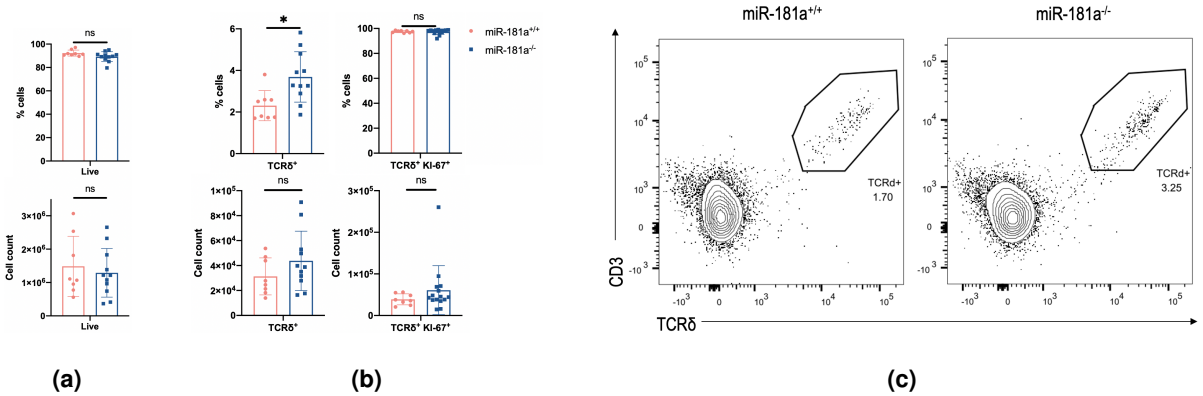


Figure 4.6: Comparison of thymic Live cell, overall γδ T cell, and KI-67^{+} γδ T cell frequency and number, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ of E17.5 mice. **(a)** Frequency and total cell number of thymic Live cells of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ E17.5 mice. **(b)** Frequency and total cell number of thymic γδ T cells and KI-67^{+} γδ T cells (Live^{+}CD45^{+}CD3^{+}TCRδ^{+}) of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ E17.5 mice. **(c)** Flow cytometry analysis of extracellular TCRδ expression in the thymus of miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) E17.5 mice. The data shown is from one experiment, consisting of n = 8 (miR-181a$^{+/+}$) and n = 11 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P $\leqslant$ 0.05 (Mann-Whitney two-tailed test).

Differences in terms of Vγ chain expression were not very pronounced in the fetal thymus of miR-181a$^{-/-}$ when compared to their littermate controls. miR-181a$^{-/-}$ mice had slightly more Vγ1-expressing

cells in terms of frequency, but not in terms of total cell numbers, and Vγ4 expression was similar between the two groups (see Figure 4.7). We thus conclude that miR-181a does not greatly affect Vγ chain expression in the fetal thymus.

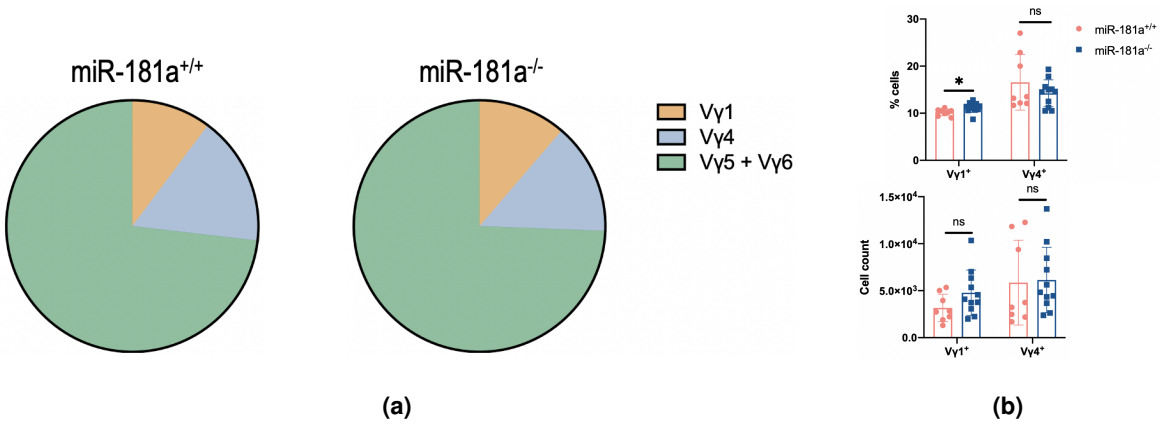


Figure 4.7: Comparison of Vγ chain usage by thymic γδ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ E17.5 mice, through extracellular fluorescent labelling of each Vγ chain and subsequent flow cytometry analysis. **(a)** Pie charts with the average proportion of thymic Vγ chain expression of miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ mice (right). **(b)** Frequency and total cell number of thymic Vγ1 and Vγ4 (CD3^{+}TCR^{+}CD24^{-}) usage of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ E17.5 mice. The data shown is from one experiment, consisting of n = 8 (miR-181a$^{+/+}$) and n = 11 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P $\leqslant$ 0.05 (Mann-Whitney two-tailed test).

Consistently, WT and miR-181a-KO mice had roughly the same cell frequencies and/or numbers for the CD44^{+}CD45RB^{+} and CD44^{-}CD45RB^{+} populations (see Figure 4.8). However, the frequency of the CD44^{hi}CD45RB^{-} population was decreased in miR-181a-KO mice, even though it is still a minor population at this stage, likely related to the fact that the wave of IL-17-producing γδ T cells begins at the embryonic day E16 [35].

Along with the drop in the CD44^{hi}CD45RB^{-} population in KO mice, a decreased frequency in terms of IL-17^{+} cells could also be seen (see Figure 4.9), although that was not observable in terms of total cell number and MFI. Interestingly, KO mice had a slight tendency for having more IFN-γ^{+} cells, and these had a significantly higher MFI.

We also looked specifically at cytokine production within populations characterized by CD44 and CD45RB (see Figure 4.10). The CD44^{hi}CD45RB^{-} population of the KO mice showed a marked decrease in the frequency of IL-17 expressing cells when compared to the WT littermate control mice, although no difference in MFI was found. As for the populations typically associated with IFN-γ production — CD44^{+}CD45RB^{+} and CD44^{-}CD45RB^{+} —, WT and KO mice had the same frequency and total cell number, but KO mice had significantly higher IFN-γ expression levels, as measured by MFI. The uncommitted CD44^{-}CD45RB^{-} subset of miR-181a$^{-/-}$ mice had a lower frequency of IL-17-producing cells, as well as decreased MFI of IL-17, when compared to miR-181a$^{+/+}$ mice. Although non-significant,

KO mice exhibited a tendency for increased frequency of IFN-γ cells, as well as a statistically significant decrease in the MFI of IFN-γ within these cells.

Despite the lack of significant differences in miR-181a expression levels in the various γδ T cell subpopulations present in E17.5 thymic cells (see Section 1.4), miR-181a seems to impact γδ T cell production and differentiation at this stage of development. To sum up, miR-181a appears to diminish γδ T cell numbers in the E17.5 thymus, and increase the frequency of IL-17-producing cells, likely from the $CD44^{hi}CD45RB^{-}$ population. Additionally, γδ T cells expressing miR-181a secrete less IFN-γ, provided by the $CD44^{+}CD45RB^{+}$, $CD44^{-}CD45RB^{+}$ and $CD44^{-}CD45RB^{-}$ populations. Based on this analysis of E17.5 thymi, we hypothesized that miR-181a promotes the differentiation of γδ17 T cells, while conversely diminishing IFN- production by γδIFN cells.

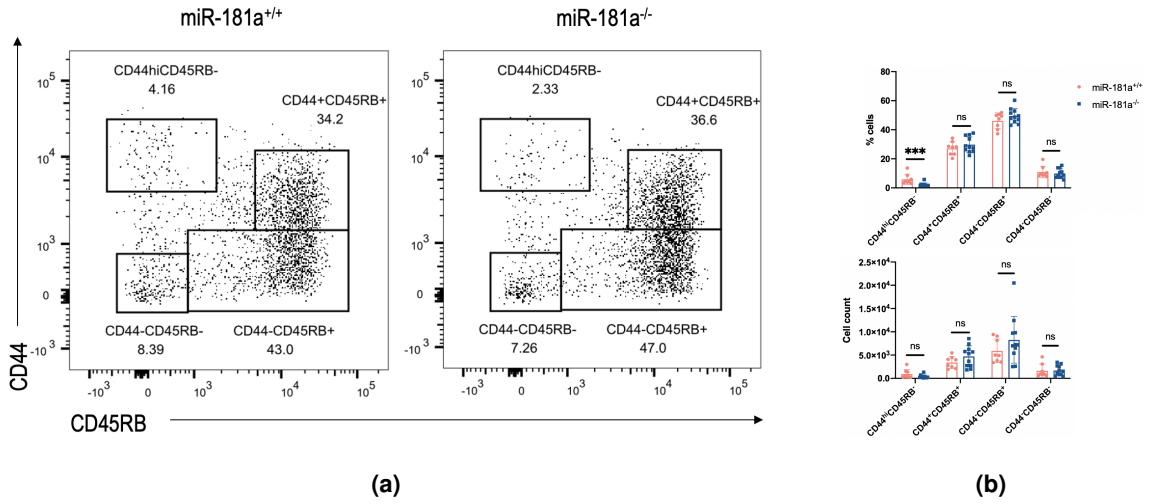


Figure 4.8: Comparison of distinct γδ T cell subsets, between miR-$181a^{+/+}$ and miR-$181a^{-/-}$ E17.5 mice, by extracellular fluorescent labelling of the CD44 and CD45RB cell surface markers and subsequent flow cytometry analysis. **(a)** Flow cytometry analysis of thymic extracellular CD44 and CD45RB expression of miR-$181a^{+/+}$ (left) and miR-$181a^{-/-}$ (right) E17.5 mice. **(b)** Frequency and total cell number of cells of four distinct thymic γδ T cell subsets, $CD44^{hi}CD45RB^{-}$, $CD44^{+}CD45RB^{+}$, $CD44^{-}CD45RB^{+}$ and $CD44^{-}CD45RB^{-}$ ($CD3^{+}TCR\delta^{+}CD24^{-}$), of miR-$181a^{+/+}$ and miR-$181a^{-/-}$ E17.5 mice. The data shown is from one experiment, consisting of $n = 8$ (miR-$181a^{+/+}$) and $n = 11$ (miR-$181a^{-/-}$). Each symbol represents an individual mouse. *** $P \leqslant 0.001$ (Mann-Whitney two-tailed test).

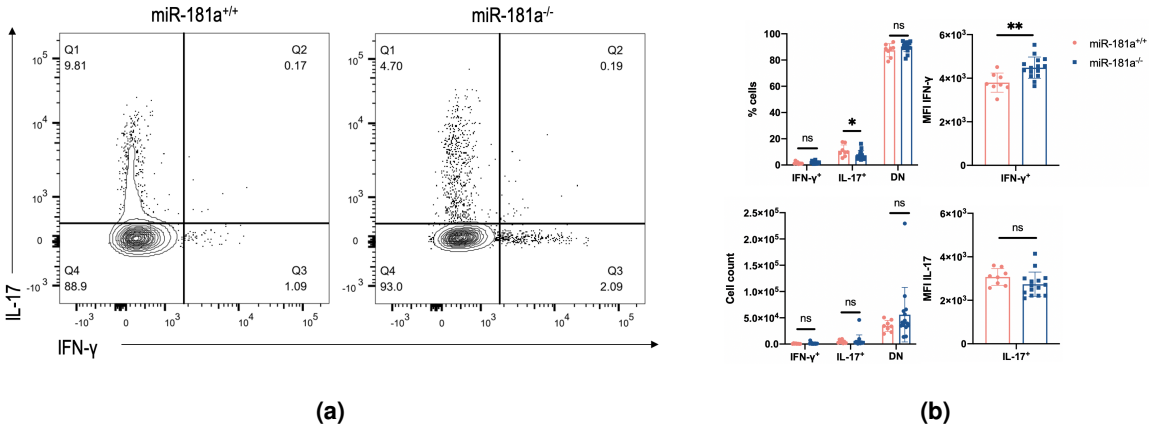
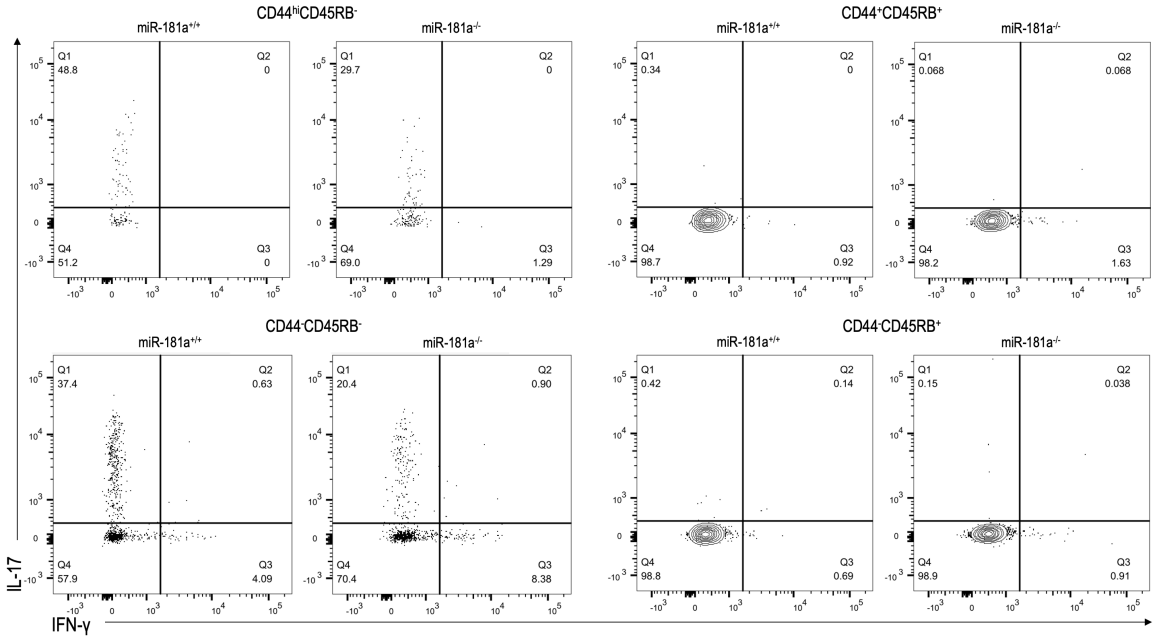
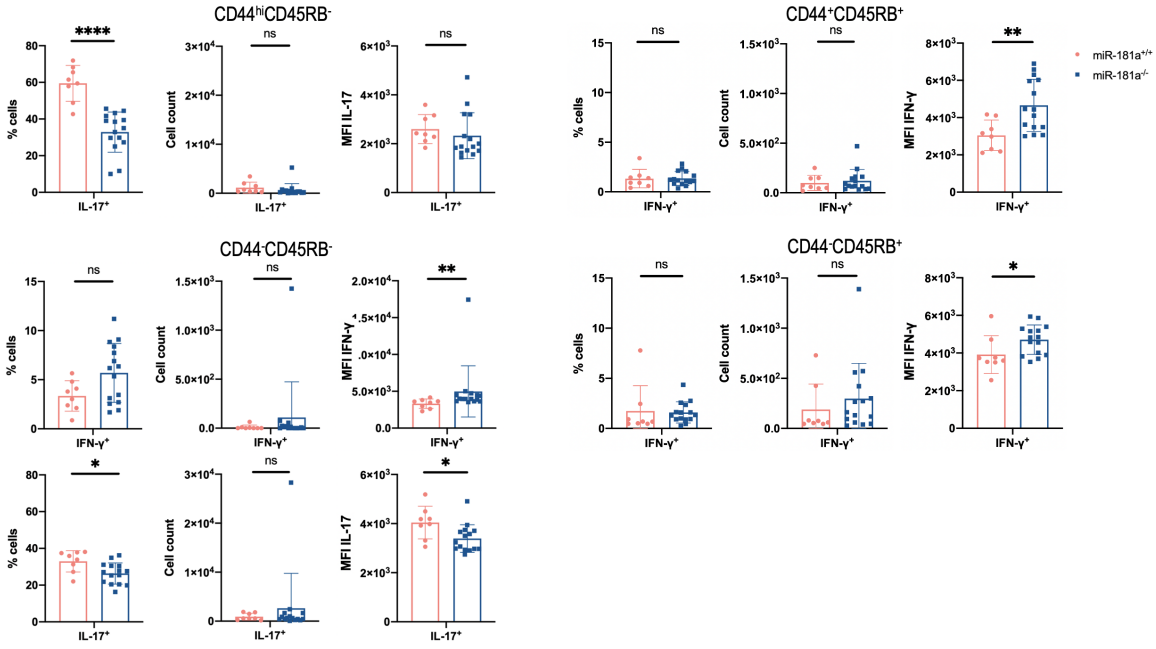


Figure 4.9: Comparison of pro-inflammatory cytokine expression by thymic γδ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ E17 mice, by intracellular fluorescent labelling of IFN-γ and IL-17 and subsequent flow cytometry analysis. **(a)** Flow cytometry analysis of intracellular IFN-γ and IL-17 expression of thymic γδ T cells of miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) E17.5 mice. **(b)** Frequency, total cell number and MFI of IFN-γ- and IL-17-expressing thymic γδ T cells (CD3^{+}TCRδ^{+}CD24^{-}) of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ E17.5 mice. The data shown is from one experiment, consisting of n = 8 (miR-181a$^{+/+}$) and n = 11 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01 (Mann-Whitney two-tailed test).



(a)



(b)

Figure 4.10: Comparison of pro-inflammatory cytokine expression by thymic γδ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ E17.5 mice, after intracellular fluorescent labelling of IFN-γ and IL-17, and surface staining of CD44 and CD45RB, and subsequent flow cytometry analysis. **(a)** Flow cytometry analysis of intracellular IFN-γ and IL-17 expression of thymic γδ T cells of miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) E17.5 mice, in γδ T cell subsets defined by labelling of CD44 and CD45RB. **(b)** Frequency, total cell number and MFI of IFN-γ- and/or IL-17-expressing thymic cells of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ of E17.5 mice, in γδ T cell subsets defined by labelling of CD44 and CD45RB. The data shown is from one experiment, consisting of n = 8 (miR-181a$^{+/+}$) and n = 11 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01, **** $\leqslant$ 0.0001 (Mann-Whitney two-tailed test).

4.1.3 *Ex vivo* analysis of D3 thymus

Having established that there were some significant differences in the fetal thymus, we wanted to see whether miR-181a also had an impact in cells developing at the neonatal phase. Additionally, the host lab had already seen that, out of all the developmental stages analyzed, miR-181a was the most differentially expressed in D3 thymic cells (see Section 1.4). Specifically, miR-181a was highly expressed in uncommitted CD44$^-$CD45RB$^-$ cells when compared to the other subpopulations, suggesting that it could play a role in pro-inflammatory γδ T cell development and differentiation. To confirm these observations, we isolated cells from the thymus of neonatal (D3) mice and did extracellular and intracellular staining for several cell surface markers and cytokines, followed by Flow Cytometry analysis (see Figure 4.1).

Contrary to the previous *ex vivo* analyses, there were fewer live leucocytes in the miR-181a$^{-/-}$ mice (see Figure 4.11(a)). We could once again observe a higher frequency of γδ T cells, but not of total cell numbers, in mice lacking miR-181a (see Figure 4.11), while the frequency of proliferating γδ T cells was similar between WT and KO mice. This suggests that miR-181a is affecting γδ T cell development of thymic D3 cells, but not their proliferation.

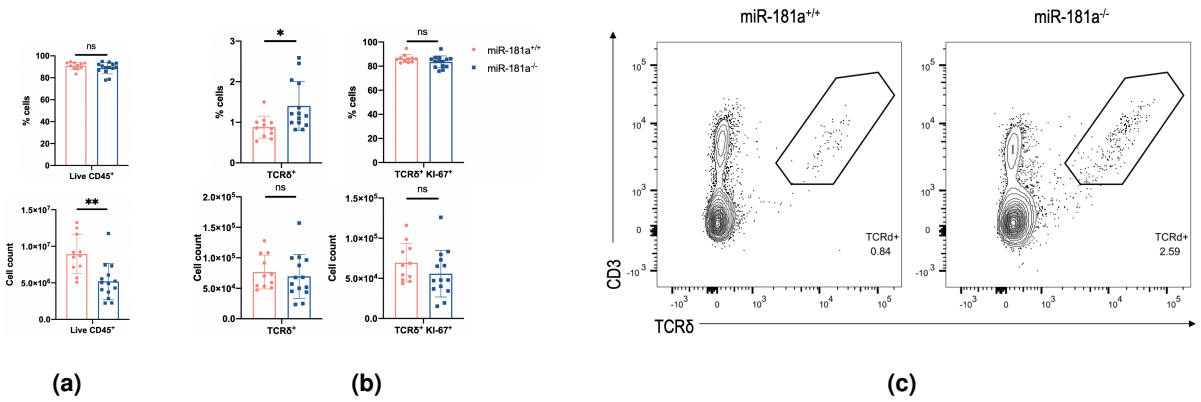


Figure 4.11: Comparison of thymic Live CD45$^+$ cell, overall γδ T cell, and KI-67$^+$ γδ T cell frequency and number of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ of D3 mice. **(a)** Frequency and total cell number of Live cells isolated from the thymus of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice. **(b)** Frequency and total cell number of γδ T cells and KI-67$^+$ γδ T cells (Live$^+$CD3$^+$TCRδ$^+$) isolated from the thymus of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice. **(c)** Flow cytometry analysis of extracellular TCRδ expression in the thymus of miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) D3 mice. The data shown is from one experiment, consisting of n $=$ 11 (miR-181a$^{+/+}$) and n $=$ 14 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01 (Mann-Whitney two-tailed test).

While probing for overall Vγ chain usage, we observed that miR-181a$^{-/-}$ mice had increased frequencies of Vγ5- and Vγ1-expressing cells, compared to miR-181a$^{+/+}$ mice, but there were no differences in terms of Vγ4 expression between the two groups (see Figure 4.12). This shows that the differences we observed in terms of Vγ1 for E17.5 mice became more pronounced at this stage, but that the differences we observed in terms of Vγ4 expression in adult mice come later in time, or impair the migration of these

cells to lymphoid organs (pLNs and spleen), or cause them to preferentially home to other tissues instead, since the developmental wave that produces Vγ4$^+$ cells begins at E18 and continues throughout life. Although Vγ5 was not analyzed at the E17.5 stage, its developmental wave starts at E12 and ends at around E17, and is associated with IFN-γ provision (see Section 1.2.2); since we observed significant differences at D3, it is likely that they were also present at E17.5, indicating that miR-181a negatively regulates Vγ1$^+$ and Vγ5$^+$ subpopulations in both the fetal and neonatal stages.

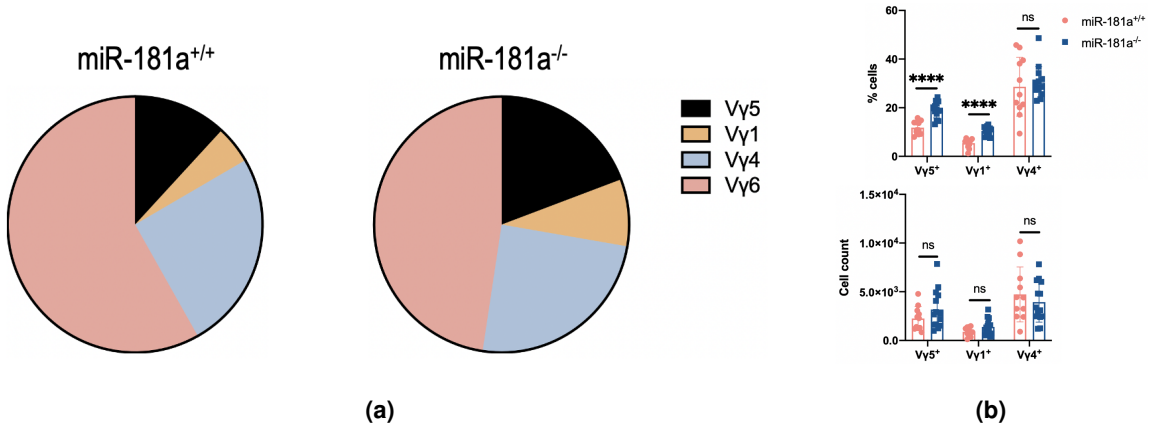


Figure 4.12: Comparison of Vγ chain usage by γδ T cells of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice through extracellular fluorescent labelling of each Vγ chain and subsequent flow cytometry analysis. **(a)** Pie charts with the average proportion of thymic Vγ chain expression of miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) mice. **(b)** Frequency and total cell number of thymic Vγ5, Vγ1 and Vγ4 (CD3$^+$TCRδ$^+$CD24$^-$) usage by miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice. The data shown is from one experiment, consisting of n = 11 (miR-181a$^{+/+}$) and n = 14 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. **** P $\leqslant$ 0.0001 (Mann-Whitney two-tailed test).

The analysis of γδ T cell subsets based on CD44 and CD45RB surface staining led us to conclude that the cell frequency of the CD44^{hi}CD45RB$^-$ population is significantly decreased in KO mice; in contrast, the CD44$^-$CD45RB$^+$ and CD44$^-$CD45RB$^-$ subsets were significantly increased (see Figure 4.13). The CD44$^+$CD45RB$^+$ subset did not differ much between WT and KO mice. Noticeably, these observations were not present at the E17.5 stage, but some of them remain consistent moving into lymphoid organs of adult mice; the ones that do being the CD44^{hi}CD45RB$^-$ and CD44$^-$CD45RB$^+$ populations, and the ones that do not being the CD44$^+$CD45RB$^+$ and CD44$^-$CD45RB$^-$ populations. However, after acquiring effector functions in the thymus, most γδ T cells move to non-lymphoid tissues [35], so perhaps some of the cells that exhibit the aforementioned differences at D3 migrated to tissues that were not analysed in this work.

We then evaluated global cytokine secretion by γδ T cells (see Figure 4.14). We saw that miR-181a$^{-/-}$ mice had more IFN-γ$^+$ and IL-17$^+$ cells than miR-181a$^{+/+}$ mice, and a higher MFI for both cytokines. KO mice had less DN cells than the WTs. Therefore, miR-181a seems to diminish IFN-γ and IL-17 production by γδ T cells in the fetal thymus.

The analysis of cytokine usage by the subsets characterized by CD44 and CD45RB expression yielded some interesting findings (see Figure 4.15). The CD44^{hi}CD45RB^{-} population of KO mice had more IL-17^{+} cells, and the CD44^{+}CD45RB^{+} and CD44^{-}CD45RB^{+} populations had more IFN-γ^{+} cells. However, differences in MFI came solely from the CD44^{-}CD45RB^{-} subset, in that KO mice had a significantly higher MFI for both cytokines in this subset.

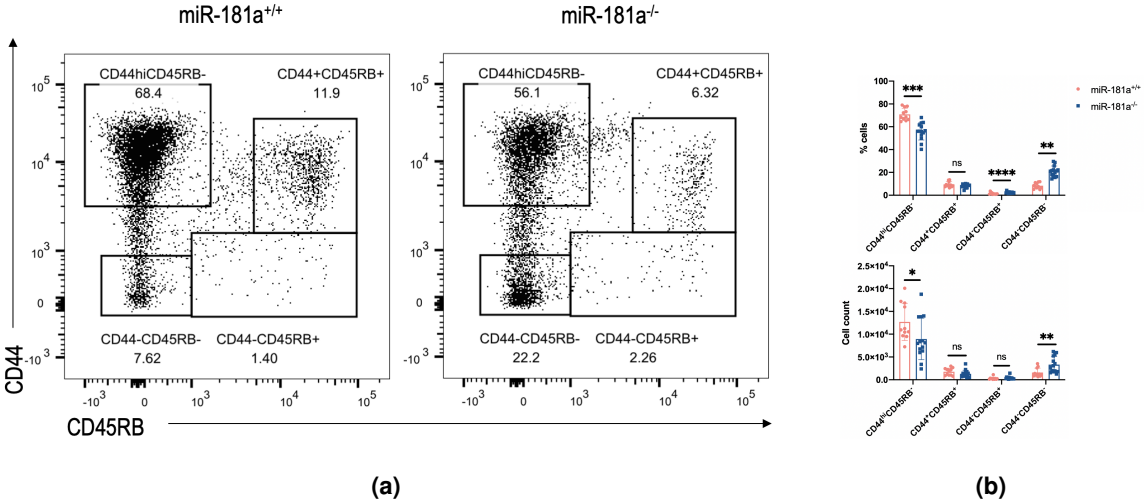


Figure 4.13: Comparison of distinct γδ T cell subsets, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice, by extracellular fluorescent labelling of the CD44 and CD45RB cell surface markers and subsequent flow cytometry analysis. **(a)** Flow cytometry analysis of thymic extracellular CD44 and CD45RB expression, between miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) D3 mice. **(b)** Frequency and total cell number of cells of four distinct thymic γδ T cell subsets, CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-}, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice. The data shown is from one experiment, consisting of n = 11 (miR-181a$^{+/+}$) and n = 14 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01, *** $\leqslant$ 0.001, **** $\leqslant$ 0.0001 (Mann-Whitney two-tailed test).

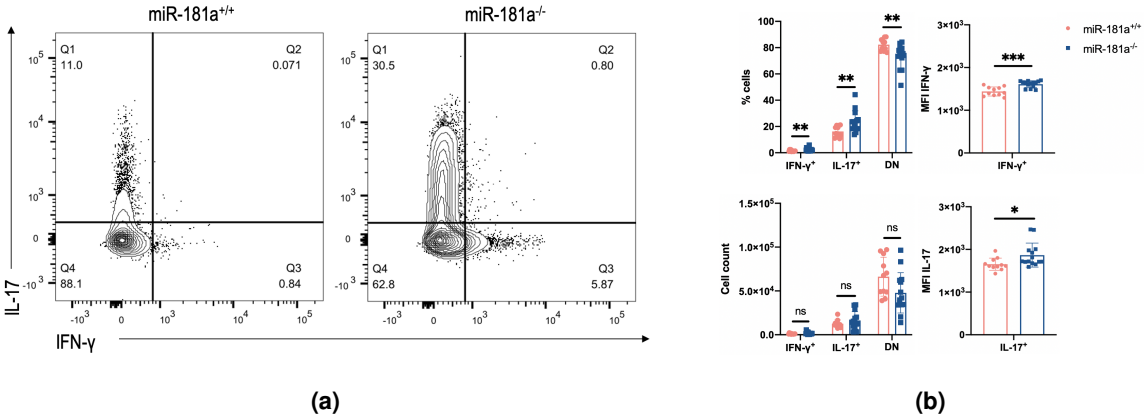
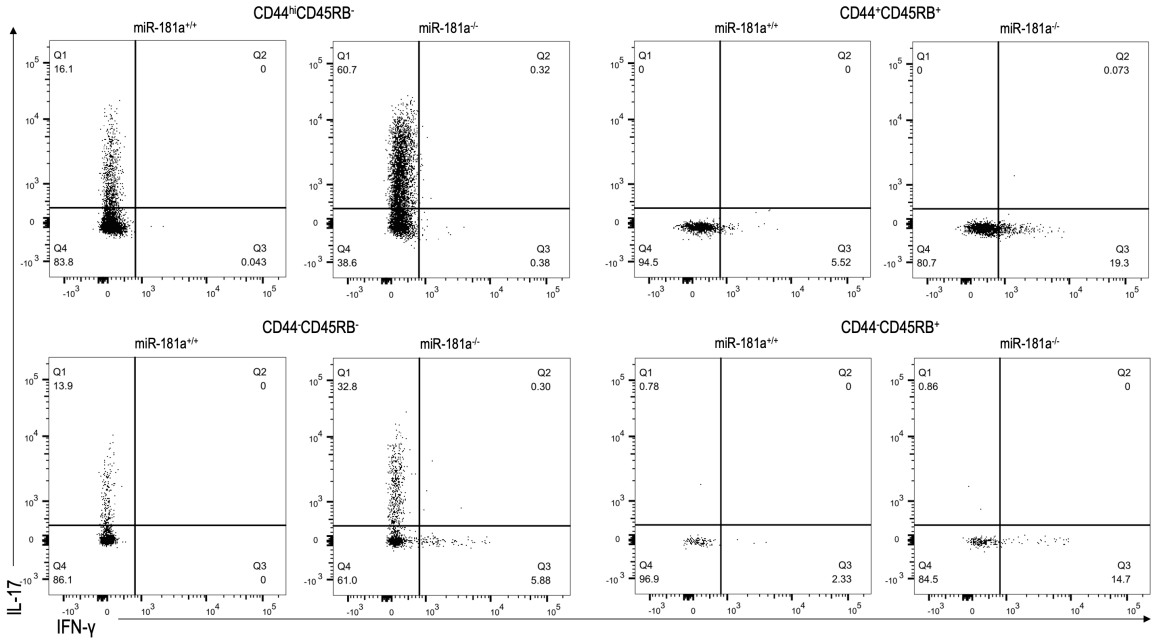
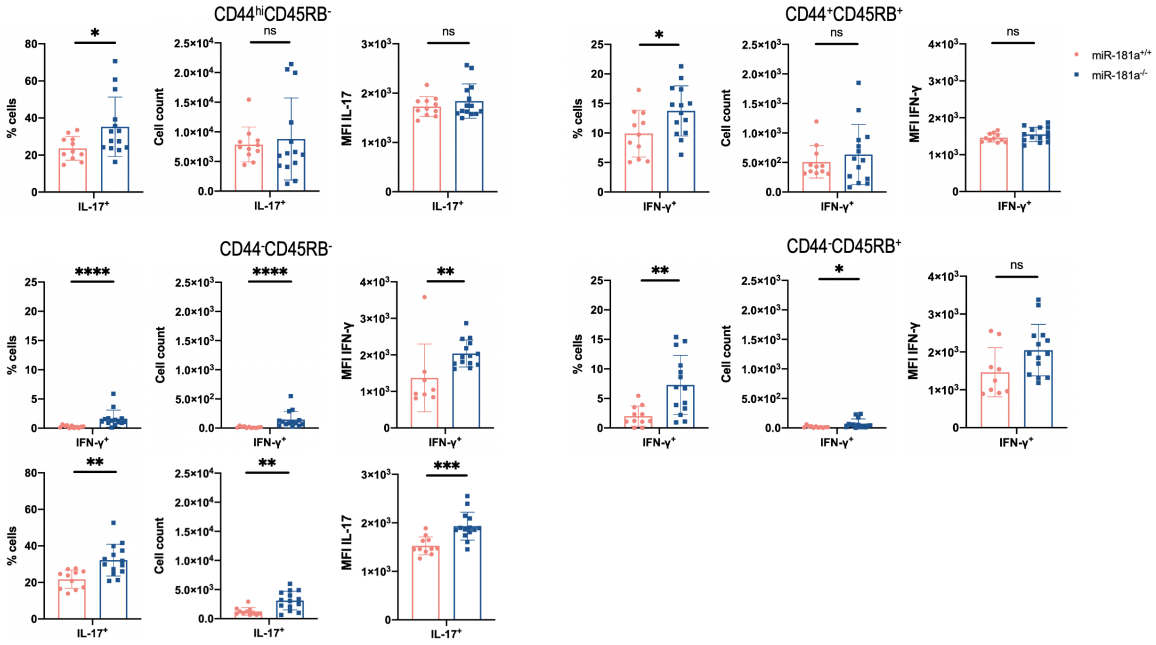


Figure 4.14: Comparison of pro-inflammatory cytokine expression by thymic γδ T cells between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice by intracellular fluorescent labelling of IFN-γ and IL-17 and subsequent flow cytometry analysis. (a) Flow cytometry analysis of intracellular IFN-γ and IL-17 expression of thymic cells of miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) D3 mice. (b) Frequency, total cell number of cells and MFI of IFN-γ- and IL-17-expressing thymic cells of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice. The data shown is from one experiment, consisting of n $=$ 11 (miR-181a$^{+/+}$) and n $=$ 14 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01, *** $\leqslant$ 0.001 (Mann-Whitney two-tailed test).



(a)



(b)

Figure 4.15: Comparison of pro-inflammatory cytokine expression by thymic γδ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice, by intracellular fluorescent labelling of IFN-γ and IL-17 and surface staining of CD44 and CD45RB, and subsequent flow cytometry analysis. **(a)** Flow cytometry analysis of intracellular IFN-γ and IL-17 expression of thymic cells, between miR-181a$^{+/+}$ (left) and miR-181a$^{-/-}$ (right) of D3 mice, in γδ T cell subsets defined by labelling of CD44 and CD45RB. **(b)** Frequency, total cell number of cells and MFI of IFN-γ- and/or IL-17-expressing thymic cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ of D3 mice, in γδ T cell subsets defined by labelling of CD44 and CD45RB. The data shown is from one experiment, consisting of n = 11 (miR-181a$^{+/+}$) and n = 14 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01, **** $\leqslant$ 0.0001 (Mann-Whitney two-tailed test).

4.2 Calcium Flux Assay

Based on the preliminary results presented in the previous sections pinpointing a potential role for miR-181a in γδ T cell differentiation and on the fact that miR-181a is a known mediator of TCR sensitivity [135], with TCR signaling strength affecting pro-inflammatory T cell commitment of γδ T cells [16,57], we sought to determine whether miR-181a played a role in γδ TCR cell signaling.

One of the hallmarks of T cell response to TCR signaling is the transient and rapid increase of calcium into the cytosol, a process mediated by Ca^{2+}-calcineurin, which constitutes one of the signaling pathways propagating the downstream signaling events caused by TCR engagement [60, 160]. miR-181a is a mediator of TCR sensitivity, known to affect the Ca^{2+} signaling pathway in αβ T cells [135]. Additionally, TCR signaling strength affects pro-inflammatory T cell commitment of γδ T cells [16, 57]. Thus, in order to test if miR-181a affects the TCR signaling of γδ T cells through the mobilization of calcium, we decided to perform a calcium flux assay (see Figure 4.16). During the assay, each sample is first supplied αCD3, which approximates the TCR chains, and then it is supplied αIgG, which crosslinks them. To assess if the results depended on the amount of αIgG given to the samples during the assay, we used two different αIgG concentrations, the lowest being 10 µg/mL, and the highest being 100 µg/mL.

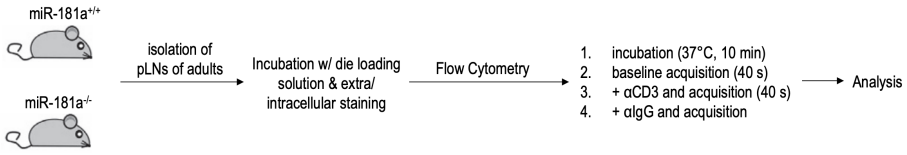


Figure 4.16: Calcium Flux assays. Cells from the pLNs of adult miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice were isolated, processed, incubated with a die loading solution, and fluorescently stained. Data was acquired in the flow cytometer, following the specified protocol.

We started by comparing calcium flux between cells expressing the TCRβ, and cells expressing the TCRδ chains (see Figure 4.17). When the lowest concentration of αIgG was added (10 µg/mL), WT and miR-181a-KO mice responded similarly, as observed by the readings of MFI PBX throughout time. Of note, PBX is a signal enhancer, which is mixed with a calcium indicator, and its measurement in the cytometer accounts for changes in calcium flux. Specifically, in the case of αβ T cells, MFI PBX did not change in neither group, whereas in γδ T cells, it suffered a slight increase throughout time in both groups of mice. When an increased concentration of αIgG was added (100 µg/mL), the response was not very pronounced either, but both αβ and γδ T cells registered a higher calcium influx overall when compared to when we added a lower concentration of αIgG, whereas a slight, but not significant, increase in cytosolic calcium was observed in miR-181a$^{-/-}$ mice, in comparison to their WT littermate control counterparts.

We then evaluated calcium flux by the γδ T cell subsets defined by the CD44 and CD45RB markers (see Figures 4.18 and 4.19). For the lowest concentration of αIgG added, all of the subsets re-

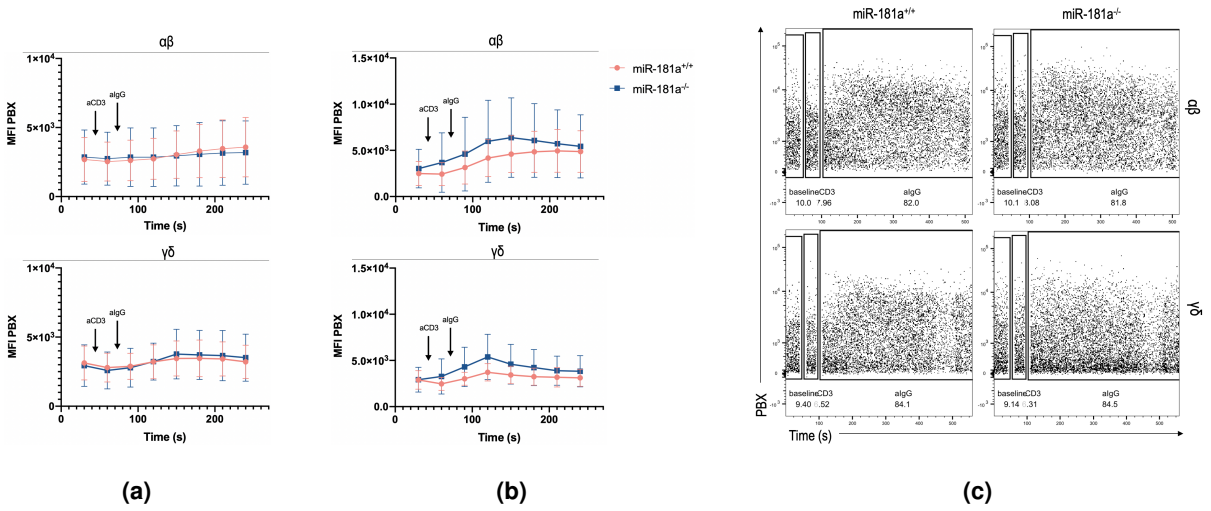
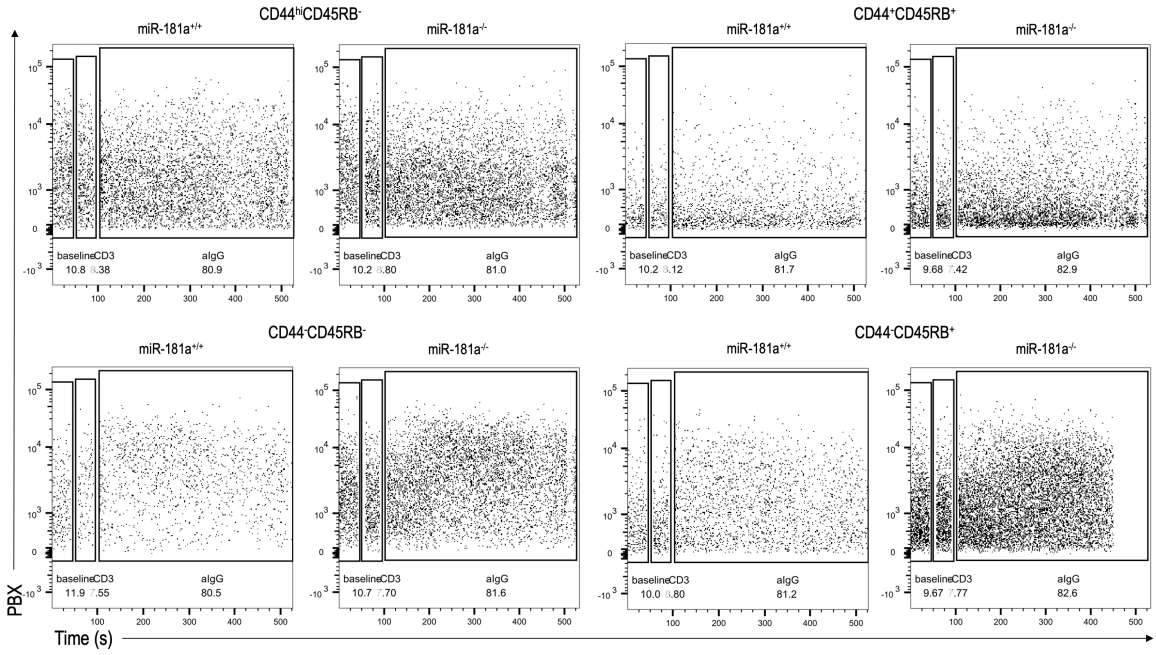


Figure 4.17: Calcium Flux Assay for αβ and γδ T cells. **(a)** Plots showing the MFI of PBX (calcium signal enhancer) throughout time, in seconds, for αβ and γδ T cells and for miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice when 10 µg/mL of aIgG were added. **(b)** Plots showing the MFI of PBX throughout time, in seconds, for αβ and γδ T cells and for miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice when 100 µg/mL of aIgG were added. **(c)** Flow cytometry analysis of intracellular PBX throughout time, in seconds, for αβ and γδ T cells, and for miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice when 100 µg/mL of aIgG were added. The data shown is from two individual experiments, each consisting of n = 2.

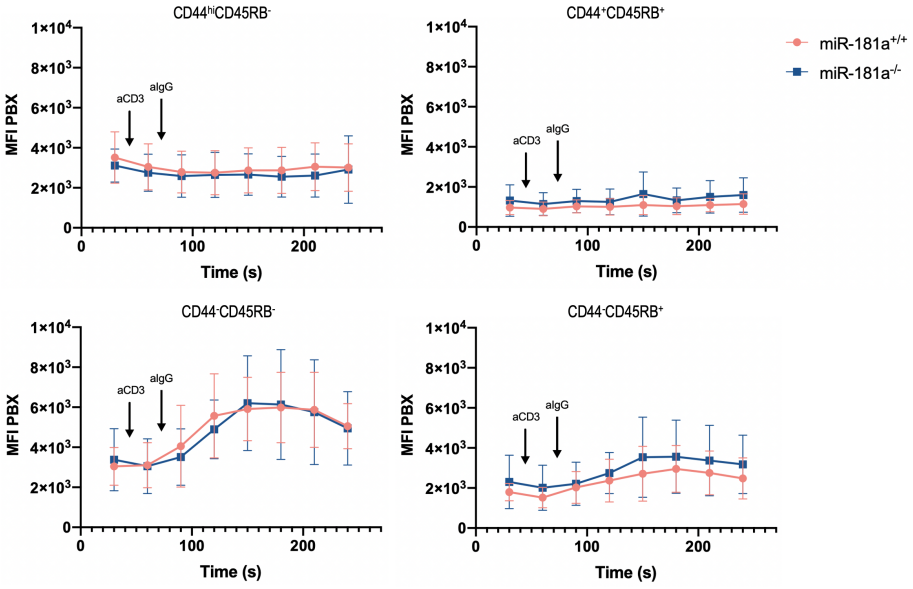
sponded similarly between miR-181a-KO and WT mice (see Figure 4.18). For the CD44^{hi}CD45RB^{-} and CD44^{+}CD45RB^{+} subsets, calcium influx did not vary throughout time; for the CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets, we observed a similar increase in the MFI of PBX for both WT and KO mice. It is noteworthy that a discrepancy in terms of cell frequency between WT and KO exists for the all of the subsets (e.g. miR-181a$^{-/-}$ have less CD44^{hi}CD45RB^{-} cells and more CD44^{-}CD45RB^{+} cells). However, this difference is inherent to each group, since it was already observable in the *ex vivo* analysis of adult lymphoid organs (see Section 4.1.1), and it does not translate into meaningful differences in terms of MFI PBX.

On the other hand, in the presence of a higher concentration of αIgG, the CD44^{hi}CD45RB^{-} population of both miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice behaves similarly. Indeed, it has been shown that only the IFN-γ "pathway" is significantly responsive to calcium flux upon TCR signaling [72], so we did not expect to see any changes for this particular subset, which is IL-17 committed. For the remaining populations — CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} —, there is a trend for miR-181a$^{-/-}$ mice to present a higher cytosolic calcium influx than the miR-181a$^{+/+}$ littermate controls, which can be observed both in the flow cytometry plots, as well as in measurements of the MFI of PBX over time, with the most pronounced changes occurring for the CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets (see Figure 4.19). The differences we observed in this case led us to conclude that 10 µg/mL of aIgG is insufficient to promote calcium influx.

These data pose the possibility that miR-181a might be implicated in the Ca^{2+} pathway of TCR signaling of IFN-γ-associated subsets of γδ T cells by augmenting TCR-mediated T cell activation.

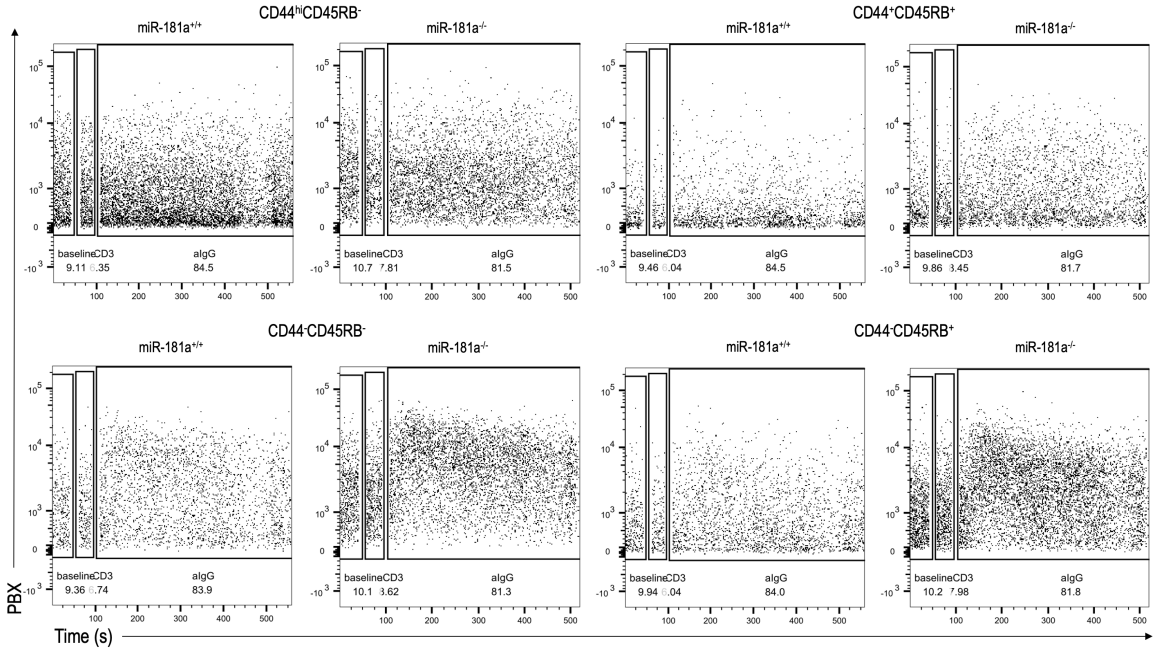


(a)

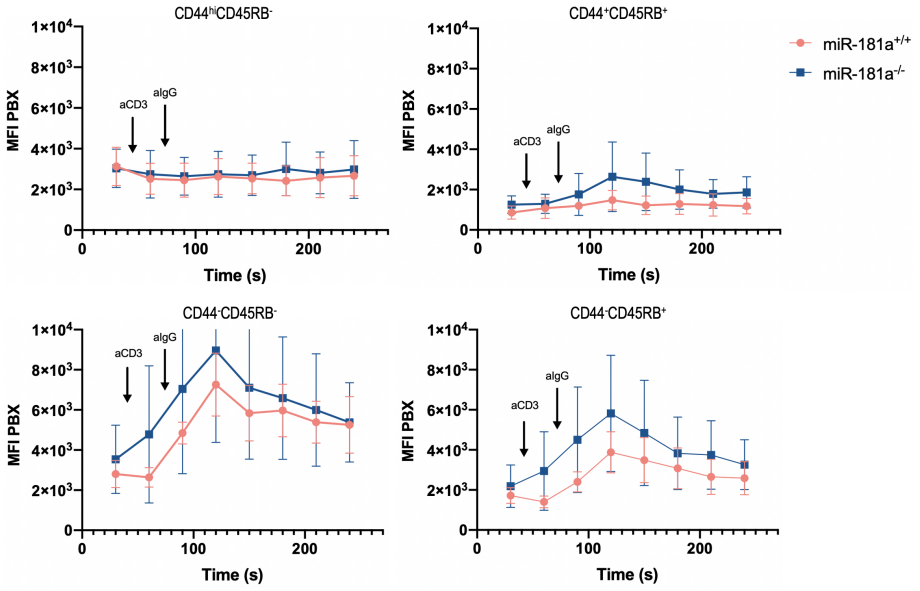


(b)

Figure 4.18: Calcium Flux Assay for the CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets of γδ T cells when 10 µg/mL of aIgG were added. **(a)** Flow cytometry analysis of intracellular PBX (calcium signal enhancer) throughout time, in seconds, for the CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets of γδ T cells, and for miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice. **(b)** Plots showing the MFI of PBX throughout time, in seconds, for the CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets of γδ T cells, and for miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice. The data shown is from two individual experiments, each consisting of n = 2.



(a)



(b)

Figure 4.19: Calcium Flux Assay for the CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets of γδ T cells when 100 µg/mL of aIgG were added. **(a)** Flow cytometry analysis of intracellular PBX (calcium signal enhancer) throughout time, in seconds, for the CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets of γδ T cells, and for miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice. **(b)** Plots showing the MFI of PBX throughout time, in seconds, for the CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets of γδ T cells, and for miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice. The data shown is from two individual experiments, each consisting of n = 2.

4.3 αCD3/αCD28 *In Vitro* Stimulation of γδ27$^-$ and γδ27$^+$ Subsets

Aiming at further dissecting the role of miR-181a on γδ T cell function, we performed an *in vitro* stimulation experiment based on a short-term TCR stimulation as described next.

As mentioned in the first chapter (see Section 1.2.2), a seminal paper by Ribot et al. described CD27 as a surface molecule that allowed for the segregation of the two pro-inflammatory γδ T cell subsets [49]. The CD27-expressing γδ T cells (γδ27$^+$) are mostly IFN-γ producers, whereas the CD27$^-$ ones (γδ27$^-$) can produce IFN-γ, but are the exclusive producers of IL-17, even though they can also produce IFN-γ, being able to become double producers (DP) under strong inflammatory conditions [56]. During TCR signaling, both the TCR and co-stimulatory molecules, such as CD28, are activated [60]. Thus, in our assay, by culturing subsets of γδ T cells in αCD3/28 overnight, these are stimulated in a way that partially mimics short-term TCR stimulation [161]. We know that γδ T cells associated with the IFN-γ "pathway" require a stronger TCR signaling to commit [57], and that miR-181a can act as a rheostat of TCR signaling strength [135]. Therefore, with this experiment we would expect to observe differences within the γδ27$^+$ subset, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice.

In this assay, we isolated peripheral γδ T cells from adult mice and sorted either γδ27$^-$ or γδ27$^+$, which we then cultured with αCD3/28 overnight, after which did intracellular fluorescent labelling of the cells, followed by Flow Cytometry analysis (see Figure 4.20). Some of the samples were analyzed for proliferation after a total of 48h in culture.

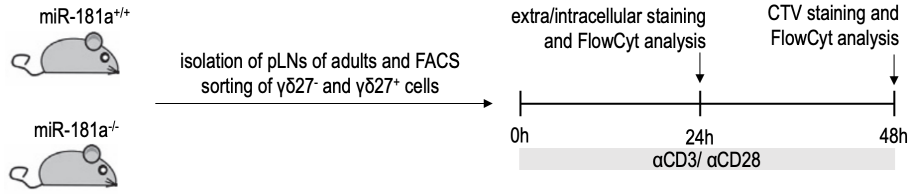


Figure 4.20: *In vitro* αCD3/αCD28 stimulation of γδ27$^-$ and γδ27$^+$ subsets. Cells were isolated from the pLNs of adult mice. They were processed and put in culture with plate-bound αCD3/αCD28. After overnight culture, some of them were extra- and intracellularly fluorescently stained and analysed by Flow Cytometry. After another 24h in culture, the cells were stained with CTV and analysed by Flow Cytometry.

By analyzing pro-inflammatory cytokine secretion by both γδ T cell subsets isolated from the pLNs of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice (see Figure 4.21), no differences in terms of MFI were observed for neither IFN-γ nor IL-17. However, for the γδ27$^+$ subset, miR-181a$^{-/-}$ mice showed a decreased frequency of IFN-γ$^+$ cells upon *in vitro* TCR stimulation. Thus, miR-181a appears to promote the frequency of γδ27$^+$ cells producing IFN-γ, despite the amount of secreted cytokine remaining unaffected.

We also looked into the proliferation of γδ27$^-$ and γδ27$^+$ cells by CellTrace Violet (CTV) expression. This reagent produces a highly stable fluorescent signal that becomes diluted upon every cell division, thus allowing for the identification of several generations of cells. There were no differences in terms of

cell proliferation between miR-181a-KO mice and their WT littermate control counterparts (see Figure 4.22).

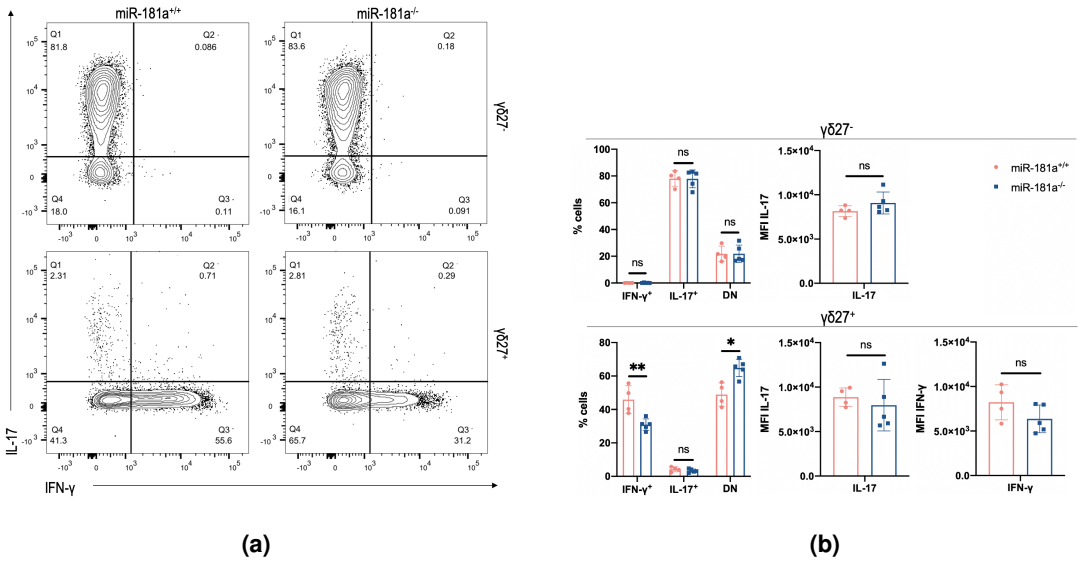


Figure 4.21: Comparison of pro-inflammatory cytokine expression by γδ27$^-$ and γδ27$^+$ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice, after αCD3/αCD28 overnight stimulation by intracellular fluorescent labelling of IFN-γ and IL-17 and subsequent flow cytometry analysis. **(a)** Flow cytometry analysis of intracellular IFN-γ and IL-17 expression by γδ27$^-$ and γδ27$^+$ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice, after αCD3/αCD28 overnight stimulation. **(b)** Frequency and MFI of IFN-γ- and IL-17-expressing γδ27$^-$ and γδ27$^+$ T cells, between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice, after αCD3/αCD28 overnight stimulation. The data shown is from two independent experiments, one consisting of n = 3 (miR-181a$^{+/+}$) and another of n = 2 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P $\leqslant$ 0.05, ** $\leqslant$ 0.01 (Mann-Whitney two-tailed test).

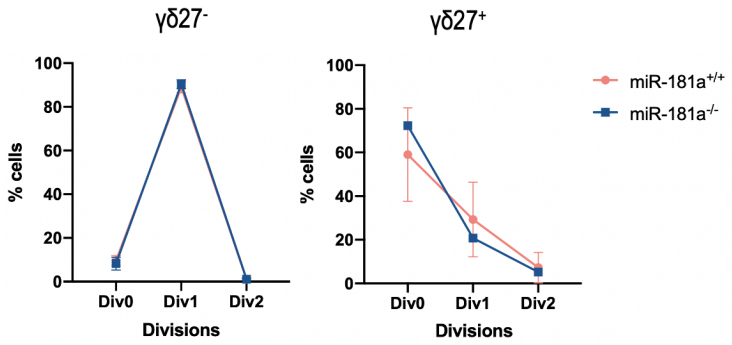


Figure 4.22: Comparison of the proliferation of γδ27$^-$ and γδ27$^+$ cells from miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice after αCD3/αCD28 overnight stimulation by intracellular CTV fluorescent labelling and subsequent flow cytometry analysis. The data shown is from two independent experiments, one consisting of n = 2 and another consisting of n = 3 (miR-181a$^{+/+}$) and n = 2 (miR-181a$^{-/-}$). Each symbol represents the average value at that division for all the mice.

4.4 Bioinformatical Target Search

Several targets of miR-181a have already been identified in the literature (see Section 1.3.2). In αβ T cells, TCR-mediated T cell activation occurs through the repression of expression of multiple inhibitory tyrosine phosphatases: SHP-2 and PTPN22, and also DUSP-5 and -6 from the ERK pathway [135, 138]. These phosphatases have been shown to inhibit TCR signaling by phosphorylation of members of the ERK pathway, or by dephosphorylation of Lck, Zap70 or CD3-associated ITAMs [60, 139]. Also, miR-181a is responsible for sustained ERK phosphorylation in Th17 cells, which leads to them having a lower threshold of activation to antigens when compared to other Th cells; *Id3* was pinpointed to be activated upon this sustained phosphorylation and to negatively regulate *Rorc* and, consequently, *Il17* [144].

The data presented so far indicates that miR-181a impacts γδ T cell development and differentiation. In particular, miR-181a was shown to promote IL-17 differentiation and to inhibit the IFN-γ pathway of γδ T cells both in fetal and adult stages. These results are consistent with a downregulation of the Ca^{2+}-Calcineurin pathway during TCR signaling, particularly in the IFN-γ-associated subsets of γδ T cells. Unlike in *ex vivo* experiments, upon short *in vitro* TCR stimulation, miR-181a appears to promote the differentiation of $CD27^+$ cells into IFN-γ^+ γδ T cells. In order to better understand the molecular/biological basis of the effects of miR-181a on γδ T cell differentiation described throughout this Thesis, we conducted a bioinformatical analysis, as to identify additional mRNA targets of miR-181a that might be involved in the context of the development and differentiation of γδ T cells.

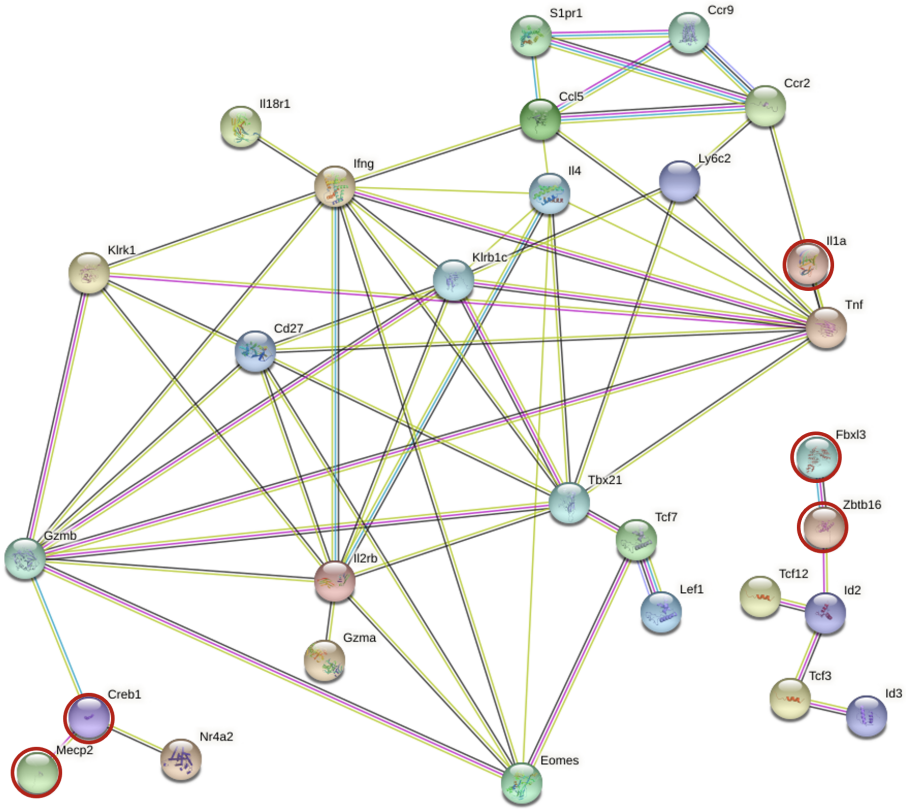
We used miRWalk3.0 to find the potential mRNA targets of miR-181a [154]. This software takes on a machine learning approach and searches within up-to-date databases to find the most likely targets of a given miRNA. It also identifies validated targets by searching within TargetScan [156]. We set the threshold for the binding probability to 0.5 and searched for possible targets within the 3' UTR region. The full list of predicted and validated targets can be found in Annex A (see Table 6.1).

Because miR-181a might be acting in both γδ T cell subsets, we decided to check for connections within the predicted/validated targets identified bioinformatically and other non-target genes that are usually associated with IFN-γ or IL-17 γδ T cell development and differentiation [49, 53, 79]. As such we built two STRING networks (Figures 4.23 and 4.24), having set the strength of interaction to a minimum of 0.7, and having hidden disconnected nodes. The full list of the manually added genes can be found in Annex A (see Table 6.2).

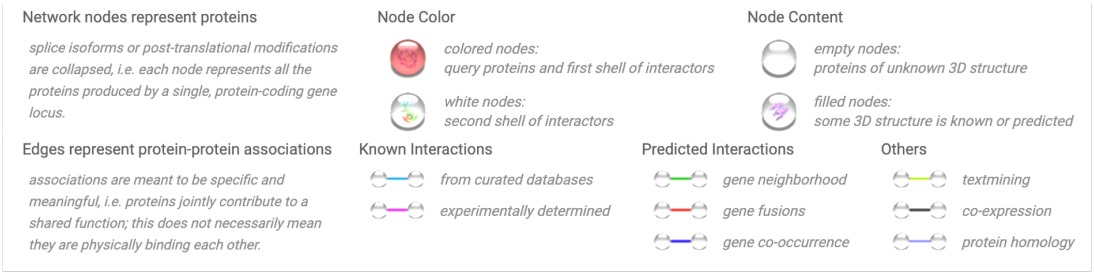
In the IFN-γ-related STRING network (see Figure 4.23(a)), we selected two interesting candidate targets: *Creb1* and *Mecp2*. *Creb1* (cAMP response element binding protein 1) is a member of the CREB/ATF family of transcription factors and is phosphorylated by protein kinase A after an intracellular increase in cAMP, activating transcription in response do cAMP, Ca^{2+} and growth factor stimulation [162, 163]. It has been identified as being directly targeted by miR-181a, affecting neuron development and synaptic plasticity [164, 165]. The interaction between miR-181a and *Creb1* has not been observed

for T cells, but Creb1 has been implicated as part of a complex, along with granzyme B and Notch2, that dynamically controls T cell cytotoxycity in $CD8^+$ cells [163]. CREB activation was also shown to play an important role in governing the IL-2 and IFN-γ production by Th1 cells [166], thus promoting Th1 responses, with IL-17 inhibiting CREB-mediated IFN-γ production [167]. Importantly, Methyl-CpG binding protein 2 (MeCP2), an X-chromosome linked nuclear protein that binds methylated DNA and has been reported to play a bifunctional role in regulating gene expression, was shown to associate with CREB1 in promoters, synergistically promoting gene expression [168]. The study of MeCP2 function in effector T cell lineages has led to the observation that MeCP2-deficient effector CD4 T cells are severely impaired in their production of inflammatory Th1 and Th17 cytokines [169].

We could not identify potential targets in the IL-17 related STRING network (see Figure 4.24(a)), since both *Il1a* and *Rorb* are promotors of the IL-17 machinery [53, 79, 144]. Given the role of these genes in promoting IL-17 production, their downregulation in the presence of miR-181a would lead to decreased IL-17 levels, which is contrary to the phenotype we observed.

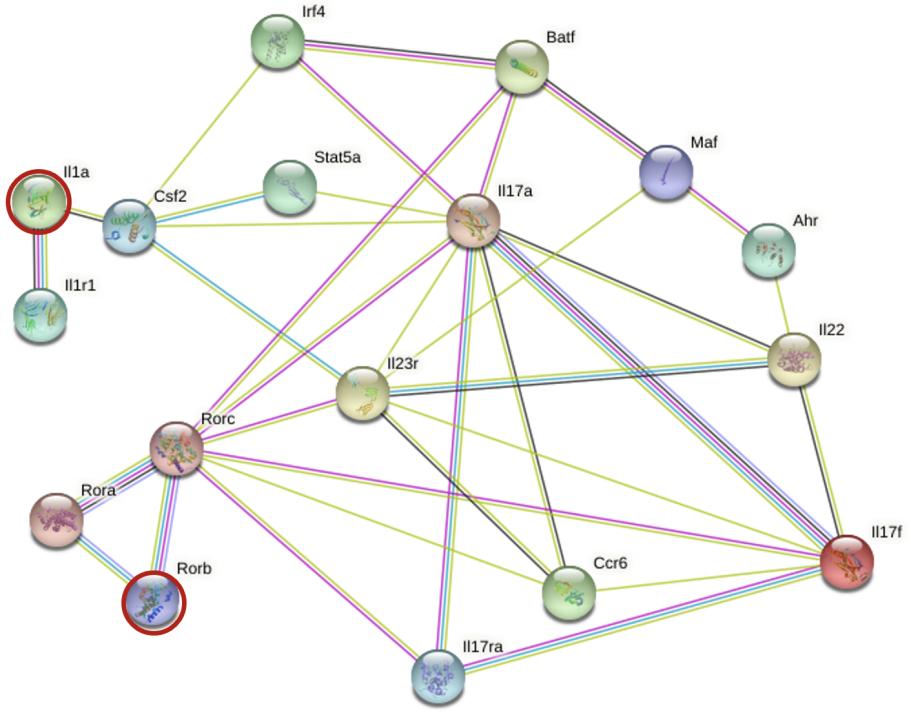


(a)

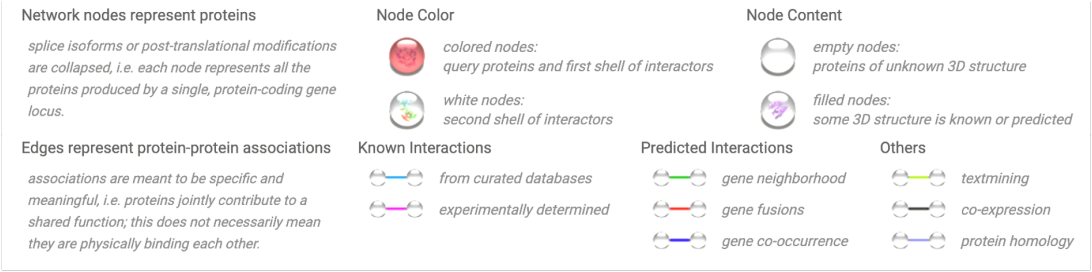


(b)

Figure 4.23: STRING network of the predicted and validated targets of miR-181a, as well as other genes related to the IFN-γ machinery of γδ T cells. Produced using [170]. **(a)** STRING network of the predicted and validated targets of miR-181a, as well as other non-target genes related to the IFN-γ machinery of γδ T cells. **(b)** Legend regarding the nodes and edges of the STRING networks. The networks were obtained with a minimum interaction score of 0.7, and disconnected nodes were hidden. The red circled nodes represent the predicted and validated target genes from our bioinformatical search, whereas the remaining nodes represent the manually added genes.



(a)



(b)

Figure 4.24: STRING network of the predicted and validated targets of miR-181a, as well as other genes related to the IL-17 machinery of γδ T cells. Produced using [170]. **(a)** STRING network of the predicted and validated targets of miR-181a, as well as other non-target genes related to the IL-17 machinery of γδ T cells. **(b)** Legend regarding the nodes and edges of the STRING networks. The networks were obtained with a minimum interaction score of 0.7, and disconnected nodes were hidden. The red circled nodes represent the predicted and validated target genes from our bioinformatical search, whereas the remaining nodes represent the manually added genes.

5

Discussion

miRNAs are a class of small non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. They have been shown to act as post-transcriptional mediators of effector function of various T cell subsets, including the $CD4^+$ T cell proinflammatory counterparts of γδ T cells, Th1 and Th17 cells. To date, however, only miR-146a has been thoroughly studied in γδ T cells [125], where it was identified to act as an IFN-γ inhibitor and to restrict γδ T cell functional plasticity *in vitro* and *in vivo*. miR-181a has been shown to be a regulator of TCR sensitivity to antigens and to promote T cell activation [135], making it a potential modulator of γδ T cell effector commitment, possibly by affecting TCR signaling strength. Although one study reports that this miRNA does not impact γδ T cell numbers in the thymus nor the periphery [153], results from the host lab showed that it was present in high amounts in the DN subset (IFN-γ^-IL-17^-) of adult peripheral γδ T cells when compared to the IFN-γ and IL-17 effector subsets, and that it was overexpressed in $CD44^-CD45RB^-$ peripheral γδ T cells of adult mice, and in thymic γδ T cells of neonatal mice (see Figure 1.4). In this work, we conducted several experiments using miR-181a-deficient mice to determine the functional role of miR-181a in γδ T cell function. We did *ex vivo* analyses of lymphoid organs of adult, fetal and neonatal mice, aiming at assessing the role of miR-181a in γδ T cell development, and we did a calcium flux assay and one *in vitro* assay, aiming at understanding the effect of miR-181a on γδ T cells upon TCR stimulation.

In this work, we established that, when compared to miR-181a-deficient mice, the presence of miR-181a led to decreased frequencies of γδ T cells at the adult (periphery), and at the fetal and neonatal (thymus) stages, while the amount of live cells remained similar (see Figures 4.2, 4.6 and 4.11).

miR-181a-sufficient fetal γδ T cells, in opposition to miR-181a-deficient γδ T cells, showed an increased frequency of $CD44^{hi}CD45RB^-$ cells, concomitant with the presence of more IL-17^+ cells. However, in terms of the relative amount of cytokine produced per a given population, we observed that IFN-γ-related populations ($CD44^+CD45RB^+$ and $CD44^-CD45RB^+$), as well as $CD44^-CD45RB^-$ have a significantly lower MFI of IFN-γ (see Figures 4.8, 4.9 and 4.10). The progression into the neonatal phase further supported these discrepancies and highlighted new phenotypes. Here, miR-181a-sufficient mice show not only increased frequencies of the IL-17-related γδ T cells — $CD44^{hi}CD45RB^-$ —, but also decreased frequencies of the immature-IFN-γ-related population — $CD44^-CD45RB^+$ —, and of the uncommitted population — $CD44^-CD45RB^-$ (see Figure 4.13). However, the presence of miR-181a led to increased levels of DN cells, and decreased levels of IFN-γ^+ and IL-17^+ cells in terms of both frequency and MFI (see Figures 4.14 and 4.15). Additionally, both Vγ5 and Vγ1 chain usage, associated with IFN-γ expression at this stage, have a lower frequency in WT mice (see Figure 4.12).

Moving into the peripheral lymphoid organs of adults, most of the differences observed in miR-$181a^{-/-}$, compared to WT cells, were in pLNs, with essentially no differences observed in the spleen. In the pLNs, there was an increased frequency of $CD44^{hi}CD45RB^-$ cells, and a decreased frequency of $CD44^-CD45RB^+$ and $CD44^-CD45RB^-$ in WT mice (see Figure 4.4). Concomitantly, there was increased

expression of Vγ4 cells, and reduced expression of Vγ1 in miR-181a-sufficient mice (see Figure 4.3). This data corroborates the hypothesis that the mechanisms occurring during development became imprinted into adulthood, since these proportions were already noticeable since the neonatal phase. A similar phenomenon has been described for the role of miR-181a in Treg cell development [147]. In this paper, miR-181a was overexpressed during thymic selection, and this expression subsided in the periphery; concurrently, the effector functions observed in the periphery had been imprinted during intrathymic differentiation. In our work, despite γδ T cell commitment becoming imprinted from the thymus to the periphery, no differences in cytokine provision by adults could be observed.

Upon TCR engagement, one of the signaling pathways used to propagate downstream signals is the Ca^{2+}-calcineutin pathway [60]. As such, one of the responses of T cells to TCR engagement is the transient and rapid influx of calcium into the cytosol [160]. Therefore, to study the the role of miR-181a within this pathway by adult γδ T cells, we performed a calcium flux assay. Although we did not observe any statistically significant differences, the IFN-γ-associated and the uncommitted populations of γδ T cells ($CD44^+CD45RB^+$, $CD44^-CD45RB^+$ and $CD44^-CD45RB^-$) of miR-181a-sufficient mice seemed to suffer decreased influx of calcium when compared to KO mice (see Figure 4.19). This could mean that miR-181a might be controlling TCR signaling strength in these subsets of γδ T cells, possibly by not allowing it to surpass a given threshold. In the *in vitro* assay, we sorted $γδ27^+$ — IFN-γ-associated — and $γδ27^-$ — IL-17-associated — cells and cultured them *in vitro* with αCD3/28, aiming to achieve a short-term TCR stimulation [49]. Within the $γδ27^+$ group, miR-$181a^{+/+}$ cells had an increased frequency of IFN-$γ^+$ cells (see Figure 4.21), indicating that miR-181a promotes the differentiation into the IFN-γ pathway upon short-term TCR stimulation.

To sum up, our data shows that miR-181a negatively affects the frequency of γδ T cells in all developmental stages. The main observation in the fetal thymus is an increased frequency of $CD44^{hi}CD45RB^+$ cells in miR-181a-sufficient mice, but no differences in the other subsets, suggesting that miR-181a is steering solely the commitment towards an IL-17 fate at that point in time. Notwithstanding, when moving towards the neonatal and adult stages, one observes not only an increased frequency of IL-17-associated γδ T cell subsets, but also a decreased frequency of IFN-γ-associated γδ T cell subsets. Furthermore, in adult cells, miR-181a might be downregulating the calcium signaling pathway upon TCR engagement; and, upon *in vitro* short-term TCR stimulation, miR-181a promotes the differentiation of DN cells of the $γδ27^+$ subset into IFN-$γ^+$ cells.

We know that miR-181a acts as a rheostat of TCR signaling strength through the modulation of several phosphatases [135]. In γδ T cells, a strong TCR signal is associated first with γδ T cell lineage commitment over an αβ one, and then, in terms of effector function acquisition, with IFN-γ commitment over IL-17 [16]. miR-181a seems to be modulating the TCR signaling threshold, controlling the frequency of γδ T cells and of its two pro-inflammatory subsets, γδIFN and γδ17, during the fetal and neonatal

stages, a phenotype which is imprinted into adulthood. At E17.5 and D3, miR-181a expression favors the γδ17 subset, while also seemingly suppressing the IFNγδ populations. One study describes miR-181a to be part of a feed-forward loop, which sustains ERK phosphorylation through the downregulation of phosphatases, which in turn maintains high levels of miR-181a expression [144]. This mechanism is evident for Th17 exclusively, in comparison to other Th cell types, and leads to a lower threshold of activation upon antigen encounter. Indeed, the preliminary studies done by the host lab disclose that miR-181a expression within the $CD44^{hi}CD45RB^{-}$ subset is the highest during fetal development, and becomes progressively lower moving into adulthood (see Figure 1.4), but a lower threshold of activation and stronger TCR signaling are incompatible with the current perspectives on proinflammatory γδ lineage commitment, where a strong TCR signal leads to an γδIFN fate, and a weak TCR signal leads to a γδ17 fate. These phenotypic observations in terms of γδ T cell commitment are consistent when moving into the periphery and adulthood. These results suggest that the effects of miR-181a expression on γδ T cells became imprinted during development, despite miR-181a overall expression subsiding in the periphery. Remarkably, these differences in commitment are not accompanied by differences in cytokine secretion in adults. Then, in response to TCR engagement, miR-181a is able to react to stronger TCR signals, "allowing" DN cells to become IFN-γ^{+}. This corroborates the previously described role for miR-181a as a modulator of TCR signalling strength throughout development and upon activation [135, 136], this time for γδ T cells. It is also possible that miR-181a has a dual effect in γδ T cell development, modulating different events depending on the pro-inflammatory subset. We propose that miR-181a expression leads to a weaker TCR signal in the fetal and neonatal thymus by targeting genes that promote a γδIFN fate, thus promoting γδ17 differentiation, a phenotype that becomes imprinted into the periphery. Then, in adults, miR-181a could mediate TCR signalling strength, affecting the differentiation of DN cells into γδIFN T cells. The proposed mechanism is summarized in Figure 5.1.

During the *ex vivo* organ analyses, we were not completely sure whether the differences observed in terms of the frequency and number of γδ T cells were inherent to γδ T cells themselves, or whether it was simply a byproduct of other biological phenomena induced by miR-181a. For instance, we already knew that miR-181a affects cell frequency and cytokine production of $CD8^{+}$ T cells and of Tregs [145, 147], and our mutant mice lacked miR-181a in all T cells. Lineage commitment by thymocytes bifurcates into either the αβ or the γδ T cell lineage [16]; as a result, if the frequency of αβ T cells is greater, the frequency of γδ T cells is smaller. To look for specific differences in the γδ T cell lineage, we would ideally use a conditional γδ T cell KO of miR-181a exclusive to γδ T cells, but no one in the research field has managed to successfully achieve a conditional γδ T cell KO. Because we sorted and cultured γδ T cells in the *in vitro* assays, we were able to analyze them without the presence of other T cells, which undoubtedly showed that miR-181a is specifically affecting γδ T cell biology.

Regarding our experimental setup, across all the experiments performed, live cells were analysed

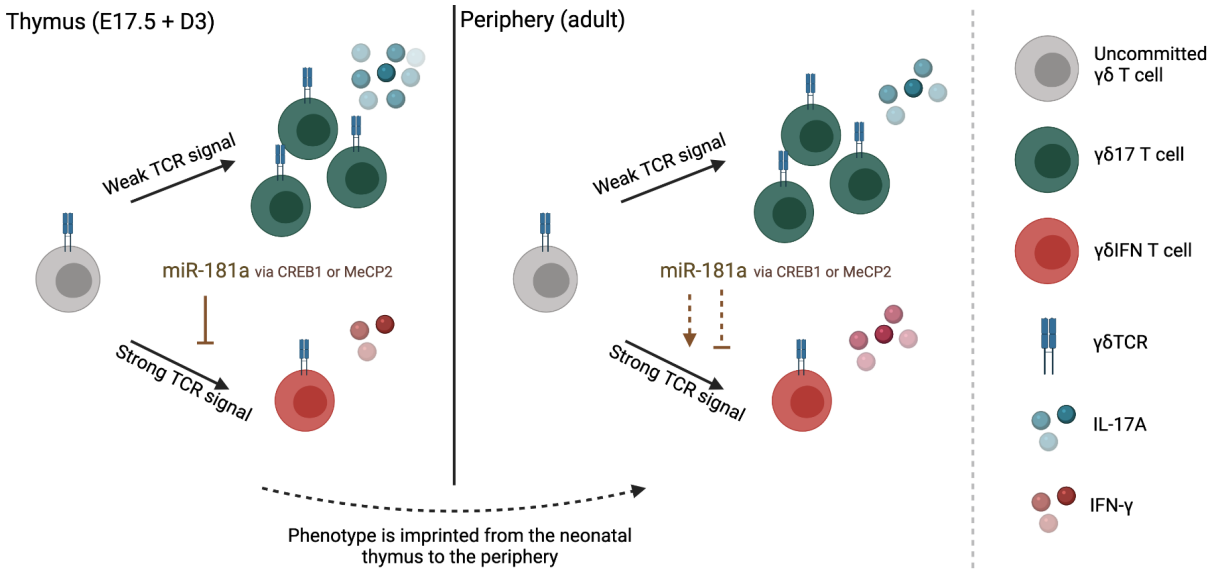


Figure 5.1: Proposed mechanism of the role of miR-181a in γδ T cell development. We propose that miR-181a expression prevents strong TCR signals in the fetal and neonatal thymus, thus promoting γδ17 differentiation. The phenotype observed in the neonatal thymus is imprinted into the periphery. Then, in adults, miR-181a could be mediator the TCR signaling response, thus affecting the differentiation of γδIFN T cells. Pro-inflammatory cytokine secretion is similar between γδIFN and γδ17 in adults. To explain our proposed mechanism, some of the possible targets of miR-181a could be CREB1 or MeCP2.

using a Live-Dead staining. However, this assay only assesses membrane integrity, so its output are exclusively dead cells, and miR-181a has been shown to induce apoptosis in various cell types by targeting anti-apoptotic genes [171, 172]. In the future, it would be interesting to use a Live-Dead/ Annexin V staining, which would allow us to differentiate between different stages of apoptosis; of note, cells staining solely for Annexin V are in an early apoptotic stage, cells staining for both Live-Dead and Annexin V are in a late apoptotic stage, and cells staining only for Live-Dead are necrotic.

Having established the cytokine levels of γδ T cells in the pLNs and spleen of adults, and since γδ T cells move mostly to non-lymphoid tissues upon thymic development [35], it would now be interesting to evaluate how cytokine provision is distributed in other tissues by γδ T cells of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice; especially, in tissues where γδ T cells play an important role in physiology and homeostasis, such as the meninges, the skin, the lungs or the intestine [173]. Additionally, the developmental wave that gives rise to Vγ4^{+} cells starts at E18 and continues throughout life. We did not observe differences in this subset between WT and miR-181a-KO mice in thymic neonatal cells, but there were more Vγ4^{+} cells in the lymphoid organs of miR-181a-sufficient adult mice. It would therefore be important to understand when and where exactly this difference began to occur, and whether any putative differences have an impact in tissues such as the lungs, the liver or the skin. One possibility is that miR-181a is affecting the migration of Vγ4 γδ T cells, since miR-181a has been reported to affect the migration of various cancer cell types [174, 175], and loss of miR-181a been associated with reduced CD4 T cell migration from the

pLNs to pathogenic sites [140].

It would be useful to perform the same *in vitro* study, sorting the γδ27^{-} and γδ27^{+} subsets and stimulating them with αCD3/28, but for a longer period of time. We observed that miR-181a positively modulated the differentiation into IFN-γ^{+} cells within the γδ27^{+} subset upon short-term TCR stimulation. It would be interesting to see whether the modulatory effect of miR-181a would still happen if the stimulation were to occur for a longer period of time. Depending on how γδ T cells respond to a long-term *in vitro* TCR stimulation, it could also be interesting to assess how miR-181a controls the balance of the two pro-inflammatory cytokines of γδ T cells upon *in vivo* challenge, since adult peripheral γδ T cells are dependent on stimuli to exert their functions [35]. To do so, many models could be used, such as infection with *Staphylococcus aureus*, to check for differences in IFN-γ provision by γδ T cells between miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice, or *Streptococcus pneumoniae*, to check for differences in IL-17 provision by γδ T cells. However, many other cells are at play in this context, which could override the effects of the interaction between miR-181a and γδ T cells.

In the future, it would also be crucial to identify the targets of miR-181a in γδ T cells. This miRNA already has a lot of known targets in other T cells. For instance, in CD8 T cells it suppresses *Id2* expression, thus limiting IFN-γ production [145], whereas it suppresses *Smad7*, inhibiting Th1 differentiation and promoting Treg differentiation [146]. miR-181a/b controls Treg development intrathymically by targeting CTLA-4 [147], and by mediating αβTCR signal strength and NAFT5 expression [148]. It modulates the priming and polarization of Th17 cells by inducing *Id3*, which negatively regulates *Rorc* and, therefore, *Il17*; this constitutes a feedback loop that leads to sustained ERK phosphorylation and increased miR-181a expression [144].

Combining our model with the results from our bioinformatical search allows us to identify a few interesting possible targets (see Annex 6.1). Our two most promising mRNA targets are CREB1 and MeCP2, that promote IFN-γ production [163, 166, 168]; therefore, targeting of either of these genes by miR-181a could suppress the commitment towards an IFN-γ lineage. In γδ T cells, the commitment to either of the two pro-inflammatory lineages likely occurs at the expense of the other, since it is postulated that strong TCR signaling leads to a γδIFN commitment, and weak TCR signaling leads to a γδ17 commitment. It is therefore possible that, if miR-181a targets either CREB1 or MeCP2 in γδ T cells, it affects both lineages in opposite ways; that is, leading to increased γδ17 commitment, and decreased γδIFN commitment. However, there are other known targets of miR-181a for other T cell types mentioned in the literature that could be influencing γδ T cell differentiation, such as DUSP5 and DUSP6, and PTPN11 and PTPN22 — a series of phosphatases that are downregulated by miR-181a in Th cells, which leads to sustained ERK phosphorylation and a lower activation threshold and stronger TCR signaling [135, 144]. Despite there being a few possible targets — CREB1, MeCP2, or even the phosphatases described in the literature —, at this point we cannot determine if any of them influence γδ T cell development and differentiation.

To understand whether any of these genes affect γδ T cells, we could start by analysing their expression levels in several γδ T cell subsets and at different developmental stages and determine whether there is an inverted correlation with miR-181a expression levels. We could also manipulate their expression levels, either inhibiting, or overexpressing them, and see if we observe an opposite phenotype, or an exacerbated one, respectively, when compared to the steady-state scenario, and whether this phenotype is the reversed to that observed for miR-181a expression manipulation. Luciferase assays could also be performed in order to determine whether miR-181a interacts directly with the CREB1 or MeCP2 3' UTR regions.

In order to robustly identify the targets of miR-181a, specifically, in γδ T cells, one possible unbiased approach would be to perform differential Argonaute 2 (Ago2) RNA Immunoprecipitation, followed by deep sequencing (Ago2 RIP-seq) [125], since Ago2 engages both miRNA and mRNA targets in miRISC, allowing for the identification miR-181a-mRNA interaction networks. However, the low numbers of γδ T cells that one is able to experimentally retrieve could pose a problem. Another possible approach would be to perform individual-nucleotide resolution UV cross-linking and immunoprecipitation (iCLIP) and characterize the miR-181 regulatory network in γδ T cells [116]. With this approach, we could compare γδ T cells of miR-181a-sufficient and -deficient mice and map the exact miR-181a target sites and identify their target sequences.

Understanding the full and accurate mechanism of miR-181a on γδ T cells could allow us to manipulate the use of γδ T cells in disease, such as psoriasis or chronic inflammation of the gingiva [173], and could possibly lead to the development of miRNA-based therapies. However, current challenges include determining the exact mRNA targets of a given miRNA, which miRNAs are relevant for a given disease, and the optimal mode of delivery of miRNA therapeutics [176].

In conclusion, in this Thesis, we have studied the role of miR-181a in the development and differentiation of the main pro-inflammatory subsets of γδ T cells. The presented findings will contribute to the knowledge of miR-181a on γδ T cell biology, thus leading to additional insights on the importance of miRNAs in the regulation of γδ T cell differentiation.

Bibliography

[1] Varadé, J., Magadán, S. & González-Fernández, Á. Human immunology and immunotherapy: main achievements and challenges. *Cellular & Molecular Immunology* 1–24 (2020).

[2] Abbas, A. K., Lichtman, A. H. & Pillai, S. *Cellular and Molecular Immunology* (Elsevier Health Sciences, 2014).

[3] Davidson, A. & Diamond, B. Autoimmune diseases. *New England Journal of Medicine* **345**, 340–350 (2001).

[4] Danilova, N. & Amemiya, C. T. Going adaptive: the saga of antibodies. *Annals of the New York Academy of Sciences* **1168**, 130–155 (2009).

[5] Rajewsky, K. Clonal selection and learning in the antibody system. *Nature* **381**, 751–758 (1996).

[6] Natoli, G. & Ostuni, R. Adaptation and memory in immune responses. *Nature Immunology* **20**, 783–792 (2019).

[7] Van Parijs, L. & Abbas, A. K. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* **280**, 243–248 (1998).

[8] Zhu, J., Yamane, H. & Paul, W. E. Differentiation of effector CD4 T cell populations. *Annual review of immunology* **28**, 445–489 (2009).

[9] Hayday, A. C. & Pennington, D. J. Key factors in the organized chaos of early T cell development. *Nature immunology* **8**, 137–144 (2007).

[10] Schatz, D. G. & Ji, Y. Recombination centres and the orchestration of V (D) J recombination. *Nature Reviews Immunology* **11**, 251–263 (2011).

[11] Jameson, S. C., Hogquist, K. A. & Bevan, M. J. Positive selection of thymocytes. *Annual review of immunology* **13**, 93–126 (1995).

[12] Klein, L., Hinterberger, M., Wirnsberger, G. & Kyewski, B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nature Reviews Immunology* **9**, 833–844 (2009).

[13] Klein, L., Kyewski, B., Allen, P. M. & Hogquist, K. A. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature Reviews Immunology* **14**, 377–391 (2014).

[14] Carding, S. R. & Egan, P. J. γδ T cells: functional plasticity and heterogeneity. *Nature reviews immunology* **2**, 336–345 (2002).

[15] Fiala, G. J., Gomes, A. Q. & Silva-Santos, B. From thymus to periphery: Molecular basis of effector γδ-T cell differentiation. *Immunological Reviews* **298**, 47–60 (2020).

[16] Sumaria, N., Martin, S. & Pennington, D. J. Developmental origins of murine γδ T-cell subsets. *Immunology* **156**, 299–304 (2019).

[17] Shibata, K. *et al.* IFN-γ–producing and IL-17–producing γδ T cells differentiate at distinct developmental stages in murine fetal thymus. *The Journal of Immunology* **192**, 2210–2218 (2014).

[18] Haas, J. D. *et al.* Development of interleukin-17-producing γδ T cells is restricted to a functional embryonic wave. *Immunity* **37**, 48–59 (2012).

[19] Havran, W. L. & Allison, J. P. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature* **335**, 443–445 (1988).

[20] Carding, S. *et al.* Developmentally regulated fetal thymic and extrathymic T-cell receptor gamma delta gene expression. *Genes & development* **4**, 1304–1315 (1990).

[21] Xiong, N., Baker, J. E., Kang, C. & Raulet, D. H. The genomic arrangement of T cell receptor variable genes is a determinant of the developmental rearrangement pattern. *Proceedings of the National Academy of Sciences* **101**, 260–265 (2004).

[22] Heilig, J. S. & Tonegawa, S. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature* **322**, 836–840 (1986).

[23] Sutoh, Y., Mohamed, R. H. & Kasahara, M. Origin and evolution of dendritic epidermal T cells. *Frontiers in immunology* **9**, 1059 (2018).

[24] Asarnow, D. M. *et al.* Limited diversity of γδ antigen receptor genes of Thy-1+ dendritic epidermal cells. *Cell* **55**, 837–847 (1988).

[25] Aono, A. *et al.* Forced expression of terminal deoxynucleotidyl transferase in fetal thymus resulted in a decrease in γδ T cells and random dissemination of vγ3vδ1 t cells in skin of newborn but not adult mice. *Immunology* **99**, 489–497 (2000).

[26] Nielsen, M. M., Witherden, D. A. & Havran, W. L. γδ T cells in homeostasis and host defence of epithelial barrier tissues. *Nature Reviews Immunology* **17**, 733–745 (2017).

[27] Sharp, L. L., Jameson, J. M., Cauvi, G. & Havran, W. L. Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. *Nature immunology* **6**, 73–79 (2005).

[28] Itohara, S. *et al.* Homing of a γδ thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. *Nature* **343**, 754–757 (1990).

[29] Ribeiro, M. *et al.* Meningeal γδ T cell–derived IL-17 controls synaptic plasticity and short-term memory. *Science immunology* **4** (2019).

[30] Minton, K. Shaping organ-specific γδ T cells. *Nature Reviews Immunology* **16**, 658–659 (2016).

[31] Grigoriadou, K., Boucontet, L. & Pereira, P. Most IL-4-producing γδ thymocytes of adult mice originate from fetal precursors. *The Journal of Immunology* **171**, 2413–2420 (2003).

[32] Pereira, P., Berthault, C., Burlen-Defranoux, O. & Boucontet, L. Critical role of TCR specificity in the development of vγ1vδ6. 3+ innate nktγδ cells. *The Journal of Immunology* **191**, 1716–1723 (2013).

[33] Pereira, P. & Boucontet, L. Innate NKT γδ and NKT αβ cells exert similar functions and compete for a thymic niche. *European journal of immunology* **42**, 1272–1281 (2012).

[34] Willcox, B. E. & Willcox, C. R. γδ TCR ligands: the quest to solve a 500-million-year-old mystery. *Nature immunology* **20**, 121–128 (2019).

[35] Silva-Santos, B., Serre, K. & Norell, H. γδ T cells in cancer. *Nature reviews immunology* **15**, 683–691 (2015).

[36] Girardi, M. *et al.* Regulation of cutaneous malignancy by γδ T cells. *Science* **294**, 605–609 (2001).

[37] Lockhart, E., Green, A. M. & Flynn, J. L. Il-17 production is dominated by γδ T cells rather than cd4 t cells during mycobacterium tuberculosis infection. *The Journal of Immunology* **177**, 4662–4669 (2006).

[38] Khairallah, C., Déchanet-Merville, J. & Capone, M. γδ T cell-mediated immunity to cytomegalovirus infection. *Frontiers in immunology* **8**, 105 (2017).

[39] Hayday, A. C. γδ T cell update: adaptate orchestrators of immune surveillance. *The Journal of Immunology* **203**, 311–320 (2019).

[40] Vantourout, P. & Hayday, A. Six-of-the-best: unique contributions of γδ T cells to immunology. *Nature Reviews Immunology* **13**, 88–100 (2013).

[41] Ribot, J. C. *et al.* Cutting edge: Adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-γ–or IL-17–producing γδ T cells upon infection. *The Journal of Immunology* **185**, 6421–6425 (2010).

[42] Hiromatsu, K. *et al.* A protective role of gamma/delta t cells in primary infection with *Listeria monocytogenes* in mice. *The Journal of experimental medicine* **175**, 49–56 (1992).

[43] Papotto, P. H., Ribot, J. C. & Silva-Santos, B. IL-17+ γδ T cells as kick-starters of inflammation. *Nature immunology* **18**, 604 (2017).

[44] Jagannathan, P. *et al.* Loss and dysfunction of vδ2+ γδ T cells are associated with clinical tolerance to malaria. *Science translational medicine* **6**, 251ra117–251ra117 (2014).

[45] Cai, Y. *et al.* Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity* **35**, 596–610 (2011).

[46] Sutton, C. E. *et al.* Interleukin-1 and IL-23 induce innate IL-17 production from γδ T cells, amplifying Th17 responses and autoimmunity. *Immunity* **31**, 331–341 (2009).

[47] Prinz, I., Silva-Santos, B. & Pennington, D. J. Functional development of γδ T cells. *European journal of immunology* **43**, 1988–1994 (2013).

[48] O'Brien, R. L. & Born, W. K. γδ T cell subsets: a link between TCR and function? In *Seminars in immunology*, vol. 22, 193–198 (Elsevier, 2010).

[49] Ribot, J. C. *et al.* CD27 is a thymic determinant of the balance between interferon-γ- and interleukin 17–producing γδ T cell subsets. *Nature immunology* **10**, 427–436 (2009).

[50] Muñoz-Ruiz, M. *et al.* TCR signal strength controls thymic differentiation of discrete proinflammatory γδ T cell subsets. *Nature immunology* **17**, 721–727 (2016).

[51] Romani, L. *et al.* Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature* **451**, 211–215 (2008).

[52] Papotto, P. H. *et al.* IL-23 drives differentiation of peripheral γδ17 T cells from adult bone marrow-derived precursors. *EMBO reports* **18**, 1957–1967 (2017).

[53] Pokrovskii, M. *et al.* Deciphering the regulatory landscape of fetal and adult γδ T-cell development at single-cell resolution. *The EMBO journal* **39**, e104159 (2020).

[54] Borst, J., Hendriks, J. & Xiao, Y. CD27 and CD70 in T cell and B cell activation. *Current opinion in immunology* **17**, 275–281 (2005).

[55] Vossen, M. T. *et al.* CD27 defines phenotypically and functionally different human NK cell subsets. *The Journal of Immunology* **180**, 3739–3745 (2008).

[56] Schmolka, N. *et al.* Epigenetic and transcriptional signatures of stable versus plastic differentiation of proinflammatory γδ T cell subsets. *Nature immunology* **14**, 1093–1100 (2013).

[57] Sumaria, N., Grandjean, C. L., Silva-Santos, B. & Pennington, D. J. Strong TCRγδ signaling prohibits thymic development of IL-17A-secreting γδ T cells. *Cell reports* **19**, 2469–2476 (2017).

[58] Siegers, G. M. *et al.* Different composition of the human and the mouse γδ T cell receptor explains different phenotypes of CD3γ and CD3δ immunodeficiencies. *Journal of Experimental Medicine* **204**, 2537–2544 (2007).

[59] Kuhns, M. S. & Badgandi, H. B. Piecing together the family portrait of TCR-CD3 complexes. *Immunological reviews* **250**, 120–143 (2012).

[60] Gaud, G., Lesourne, R. & Love, P. E. Regulatory mechanisms in T cell receptor signalling. *Nature Reviews Immunology* **18**, 485–497 (2018).

[61] Ribeiro, S. T., Ribot, J. C. & Silva-Santos, B. Five layers of receptor signalling in γδ T cell differentiation and activation. *Frontiers in immunology* **6**, 15 (2015).

[62] Turchinovich, G. & Pennington, D. J. T cell receptor signalling in γδ cell development: strength isn't everything. *Trends in immunology* **32**, 567–573 (2011).

[63] Ciofani, M. & Zúñiga-Pflücker, J. C. Determining γδ versus αβ T cell development. *Nature Reviews Immunology* **10**, 657–663 (2010).

[64] Haks, M. C. *et al.* Attenuation of γδTCR signaling efficiently diverts thymocytes to the αβ lineage. *Immunity* **22**, 595–606 (2005).

[65] Hayes, S. M. & Love, P. E. Distinct structure and signaling potential of the γδTCR complex. *Immunity* **16**, 827–838 (2002).

[66] Lauritsen, J. P. H. *et al.* Marked induction of the helix-loop-helix protein id3 promotes the γδ T cell fate and renders their functional maturation Notch independent. *Immunity* **31**, 565–575 (2009).

[67] Lee, S.-Y. *et al.* Noncanonical mode of ERK action controls alternative αβ and γδ T cell lineage fates. *Immunity* **41**, 934–946 (2014).

[68] Jensen, K. D. *et al.* Thymic selection determines γδ T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon γ. *Immunity* **29**, 90–100 (2008).

[69] Turchinovich, G. & Hayday, A. C. Skint-1 identifies a common molecular mechanism for the development of interferon-γ-secreting versus interleukin-17-secreting γδ T cells. *Immunity* **35**, 59–68 (2011).

[70] Barbee, S. D. *et al.* Skint-1 is a highly specific, unique selecting component for epidermal T cells. *Proceedings of the National Academy of Sciences* **108**, 3330–3335 (2011).

[71] Coffey, F. *et al.* The TCR ligand-inducible expression of cd73 marks γδ lineage commitment and a metastable intermediate in effector specification. *Journal of Experimental Medicine* **211**, 329–343 (2014).

[72] Wencker, M. *et al.* Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. *Nature immunology* **15**, 80–87 (2014).

[73] DeBarros, A., Chaves-Ferreira, M., d'Orey, F., Ribot, J. C. & Silva-Santos, B. CD70–CD27 interactions provide survival and proliferative signals that regulate t cell receptor-driven activation of human γδ peripheral blood lymphocytes. *European journal of immunology* **41**, 195–201 (2011).

[74] Yin, Z. *et al.* T-bet expression and failure of GATA-3 cross-regulation lead to default production of IFN-γ by γδ T cells. *The Journal of Immunology* **168**, 1566–1571 (2002).

[75] Szabo, S. J. *et al.* Distinct effects of T-bet in T_H1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science* **295**, 338–342 (2002).

[76] Suto, A., Wurster, A. L., Reiner, S. L. & Grusby, M. J. IL-21 inhibits IFN-γ production in developing Th1 cells through the repression of eomesodermin expression. *The Journal of Immunology* **177**, 3721–3727 (2006).

[77] Barros-Martins, J. *et al.* Effector γδ T cell differentiation relies on master but not auxiliary Th cell transcription factors. *The Journal of Immunology* **196**, 3642–3652 (2016).

[78] Lino, C. N., Barros-Martins, J., Oberdörfer, L., Walzer, T. & Prinz, I. Eomes expression reports the progressive differentiation of IFN-γ-producing Th1-like γδ T cells. *European journal of immunology* **47**, 970–981 (2017).

[79] Malhotra, N. *et al.* A network of high-mobility group box transcription factors programs innate interleukin-17 production. *Immunity* **38**, 681–693 (2013).

[80] Ivanov, I. I. *et al.* The orphan nuclear receptor rorγt directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* **126**, 1121–1133 (2006).

[81] Lochner, M. *et al.* In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ rorγt+ T cells. *Journal of Experimental Medicine* **205**, 1381–1393 (2008).

[82] Laird, R. M., Laky, K. & Hayes, S. M. Unexpected role for the B cell-specific Src family kinase B lymphoid kinase in the development of IL-17–producing γδ T cells. *The Journal of Immunology* **185**, 6518–6527 (2010).

[83] Raifer, H. *et al.* Unlike αβ T cells, γδ T cells, LT i cells and NKT cells do not require IRF 4 for the production of IL-17A and IL-22. *European journal of immunology* **42**, 3189–3201 (2012).

[84] McKenzie, D. R. *et al.* IL-17-producing γδ T cells switch migratory patterns between resting and activated states. *Nature communications* **8**, 1–13 (2017).

[85] Shibata, K. *et al.* Notch-Hes1 pathway is required for the development of IL-17–producing γδ T cells. *Blood, The Journal of the American Society of Hematology* **118**, 586–593 (2011).

[86] Kadekar, D. *et al.* The neonatal microenvironment programs innate γδ T cells through the transcription factor STAT5. *Journal of Clinical Investigation* **130**, 2496–2508 (2020).

[87] Gray, E. E. *et al.* Deficiency in IL-17-committed V γ 4+ γδ T cells in a spontaneous Sox13-mutant CD45. 1+ congenic mouse substrain provides protection from dermatitis. *Nature immunology* **14**, 584–592 (2013).

[88] Inácio, D. P., Amado, T., Silva-Santos, B. & Gomes, A. Q. Control of T cell effector functions by miRNAs. *Cancer letters* **427**, 63–73 (2018).

[89] Ambros, V. The functions of animal microRNAs. *Nature* **431**, 350–355 (2004).

[90] Neilsen, C. T., Goodall, G. J. & Bracken, C. P. IsomiRs–the overlooked repertoire in the dynamic microRNAome. *Trends in genetics* **28**, 544–549 (2012).

[91] Boyd, S. D. Everything you wanted to know about small RNA but were afraid to ask. *Laboratory investigation* **88**, 569–578 (2008).

[92] Krol, J., Loedige, I. & Filipowicz, W. The widespread regulation of microRNA biogenesis, function and decay. *Nature Reviews Genetics* **11**, 597–610 (2010).

[93] Jonas, S. & Izaurralde, E. Towards a molecular understanding of microRNA-mediated gene silencing. *Nature reviews genetics* **16**, 421–433 (2015).

[94] Monticelli, S. *et al.* MicroRNA profiling of the murine hematopoietic system. *Genome biology* **6**, 1–15 (2005).

[95] Chong, M. M., Rasmussen, J. P., Rudensky, A. Y. & Littman, D. R. The RNAseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease. *The Journal of experimental medicine* **205**, 2005–2017 (2008).

[96] Muljo, S. A. *et al.* Aberrant T cell differentiation in the absence of dicer. *The Journal of experimental medicine* **202**, 261–269 (2005).

[97] Amado, T., Schmolka, N., Metwally, H., Silva-Santos, B. & Gomes, A. Q. Cross-regulation between cytokine and microRNA pathways in T cells. *European journal of immunology* **45**, 1584–1595 (2015).

[98] O'Connell, R. M. *et al.* MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* **33**, 607–619 (2010).

[99] Banerjee, A., Schambach, F., DeJong, C. S., Hammond, S. M. & Reiner, S. L. Micro-RNA-155 inhibits IFN-γ signaling in CD4+ T cells. *European journal of immunology* **40**, 225–231 (2010).

[100] Soond, D. R. *et al.* PI3K p110δ regulates T-cell cytokine production during primary and secondary immune responses in mice and humans. *Blood, The Journal of the American Society of Hematology* **115**, 2203–2213 (2010).

[101] Handi, J., Patterson, S. J. & Levings, M. The role of the PI3K signaling pathway in CD4+ T cell differentiation and function. *Frontiers in immunology* **3**, 245 (2012).

[102] Wu, T. *et al.* Cutting edge: miR-17-92 is required for both CD4 Th1 and T follicular helper cell responses during viral infection. *The Journal of Immunology* **195**, 2515–2519 (2015).

[103] Jiang, S. *et al.* Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. *Blood* **118**, 5487–5497 (2011).

[104] Rezaei, N. *et al.* MicroRNA-92a drives Th1 responses in the experimental autoimmune encephalomyelitis. *Inflammation* **42**, 235–245 (2019).

[105] Steiner, D. F. *et al.* MicroRNA-29 regulates t-box transcription factors and interferon-γ production in helper T cells. *Immunity* **35**, 169–181 (2011).

[106] Smith, K. M. *et al.* miR-29ab1 deficiency identifies a negative feedback loop controlling Th1 bias that is dysregulated in multiple sclerosis. *The Journal of Immunology* **189**, 1567–1576 (2012).

[107] Chandiran, K. *et al.* Notch1 primes CD4 T cells for T helper type I differentiation through its early effects on miR-29. *Molecular immunology* **99**, 191–198 (2018).

[108] Wang, J., Yin, J., Peng, H. & Liu, A. MicroRNA-29 mediates anti-inflammatory effects and alleviation of allergic responses and symptoms in mice with allergic rhinitis. *Allergy, Asthma & Clinical Immunology* **17**, 1–11 (2021).

[109] Ma, F. *et al.* The microrna mir-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-γ. *Nature immunology* **12**, 861–869 (2011).

[110] Horita, M., Farquharson, C. & Stephen, L. A. The role of miR-29 family in disease. *Journal of Cellular Biochemistry* (2021).

[111] Du, C. *et al.* MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. *Nature immunology* **10**, 1252 (2009).

[112] Liu, Y. *et al.* Elevated microRNA-326 levels regulate the IL-23/IL-23R/Th17 cell axis in Hashimoto's thyroiditis by targeting a disintegrin and metalloprotease 17. *Thyroid* **30**, 1327–1337 (2020).

[113] Vega-Cárdenas, M. *et al.* Increased levels of adipose tissue-resident Th17 cells in obesity associated with miR-326. *Immunology letters* **211**, 60–67 (2019).

[114] Qu, X. *et al.* MiR-384 regulates the Th17/Treg ratio during experimental autoimmune encephalomyelitis pathogenesis. *Frontiers in cellular neuroscience* **11**, 88 (2017).

[115] Han, J. *et al.* STAT3 regulates miR-384 transcription during Th17 polarization. *Frontiers in cell and developmental biology* **7**, 253 (2019).

[116] Hsin, J.-P., Lu, Y., Loeb, G. B., Leslie, C. S. & Rudensky, A. Y. The effect of cellular context on miR-155-mediated gene regulation in four major immune cell types. *Nature immunology* **19**, 1137–1145 (2018).

[117] Xu, M. *et al.* MiR-155 contributes to Th17 cells differentiation in dextran sulfate sodium (DSS)-induced colitis mice via Jarid2. *Biochemical and biophysical research communications* **488**, 6–14 (2017).

[118] Zhu, F. *et al.* miR-155 antagomir protect against DSS-induced colitis in mice through regulating Th17/Treg cell balance by Jarid2/Wnt/β-catenin. *Biomedicine & Pharmacotherapy* **126**, 109909 (2020).

[119] Wang, D. *et al.* MiRNA-155 regulates the Th17/Treg ratio by targeting SOCS1 in severe acute pancreatitis. *Frontiers in physiology* **9**, 686 (2018).

[120] Kästle, M. *et al.* micro RNA cluster 106a˜363 is involved in T helper 17 cell differentiation. *Immunology* **152**, 402–413 (2017).

[121] Sanctuary, M. R. *et al.* miR-106a deficiency attenuates inflammation in murine IBD models. *Mucosal immunology* **12**, 200–211 (2019).

[122] Mikami, Y. *et al.* MicroRNA-221 and-222 modulate intestinal inflammatory Th17 cell response as negative feedback regulators downstream of interleukin-23. *Immunity* (2021).

[123] Cobb, B. S. *et al.* A role for Dicer in immune regulation. *The Journal of experimental medicine* **203**, 2519–2527 (2006).

[124] Haas, J. D. *et al.* Expression of miRNAs miR-133b and miR-206 in the Il17a/f locus is co-regulated with IL-17 production in $\alpha\beta$ and $\gamma\delta$ T cells. *PloS one* **6**, e20171 (2011).

[125] Schmolka, N. *et al.* MicroRNA-146a controls functional plasticity in $\gamma\delta$ T cells by targeting NOD1. *Science immunology* **3** (2018).

[126] Dolens, A.-C. *et al.* Distinct Notch1 and BCL11B requirements mediate human $\gamma\delta/\alpha\beta$ T cell development. *EMBO reports* **21**, e49006 (2020).

[127] Zhu, Y. *et al.* miR-125b-5p and miR-99a-5p downregulate human $\gamma\delta$ T-cell activation and cytotoxicity. *Cellular & molecular immunology* **16**, 112–125 (2019).

[128] Yang, Z. *et al.* Evolution of the mir-181 microRNA family. *Computers in biology and medicine* **52**, 82–87 (2014).

[129] Chen, C.-Z., Li, L., Lodish, H. F. & Bartel, D. P. MicroRNAs modulate hematopoietic lineage differentiation. *science* **303**, 83–86 (2004).

[130] Ryan, D. G., Oliveira-Fernandes, M., Lavker, R. M. *et al.* MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity. *Mol Vis* **12**, 1175–1184 (2006).

[131] Miska, E. A. *et al.* Microarray analysis of microRNA expression in the developing mammalian brain. *Genome biology* **5**, 1–13 (2004).

[132] Naguibneva, I. *et al.* The microrna miR-181 targets the homeobox protein Hox-a11 during mammalian myoblast differentiation. *Nature cell biology* **8**, 278–284 (2006).

[133] Pop-Bica, C. *et al.* MiR-181 family-specific behavior in different cancers: a meta-analysis view. *Cancer and Metastasis Reviews* **37**, 17–32 (2018).

[134] Neilson, J. R., Zheng, G. X., Burge, C. B. & Sharp, P. A. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes & development* **21**, 578–589 (2007).

[135] Li, Q.-J. *et al.* miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* **129**, 147–161 (2007).

[136] Ebert, P. J., Jiang, S., Xie, J., Li, Q.-J. & Davis, M. M. An endogenous positively selecting peptide enhances mature T cell responses and becomes an autoantigen in the absence of microRNA miR-181a. *Nature immunology* **10**, 1162 (2009).

[137] Fragoso, R. *et al.* Modulating the strength and threshold of NOTCH oncogenic signals by mir-181a-1/b-1. *PLoS Genet* **8**, e1002855 (2012).

[138] Lindsay, M. A. microRNAs and the immune response. *Trends in immunology* **29**, 343–351 (2008).

[139] Wu, J. *et al.* Identification of substrates of human protein-tyrosine phosphatase PTPN22. *Journal of Biological Chemistry* **281**, 11002–11010 (2006).

[140] Schaffert, S. A. *et al.* mir-181a-1/b-1 modulates tolerance through opposing activities in selection and peripheral T cell function. *The Journal of Immunology* **195**, 1470–1479 (2015).

[141] Li, G. *et al.* Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. *Nature medicine* **18**, 1518–1524 (2012).

[142] Ye, Z. *et al.* Regulation of miR-181a expression in T cell aging. *Nature communications* **9**, 1–11 (2018).

[143] Salmond, R. J., Brownlie, R. J., Morrison, V. L. & Zamoyska, R. The tyrosine phosphatase PTPN22 discriminates weak self peptides from strong agonist TCR signals. *Nature immunology* **15**, 875–883 (2014).

[144] Mele, F. *et al.* ERK phosphorylation and miR-181a expression modulate activation of human memory T_H17 cells. *Nature communications* **6**, 1–12 (2015).

[145] Amado, T. *et al.* MicroRNA-181a regulates IFN-γ expression in effector CD8+ T cell differentiation. *Journal of Molecular Medicine* **98**, 309–320 (2020).

[146] Ghorbani, S. *et al.* MicroRNA-181 variants regulate T cell phenotype in the context of autoimmune neuroinflammation. *Frontiers in immunology* **8**, 758 (2017).

[147] Łyszkiewicz, M. *et al.* miR-181a/b-1 controls thymic selection of Treg cells and tunes their suppressive capacity. *PLoS biology* **17**, e2006716 (2019).

[148] Serr, I. *et al.* A miRNA181a/NFAT5 axis links impaired T cell tolerance induction with autoimmune type 1 diabetes. *Science translational medicine* **10** (2018).

[149] Mattner, J. *et al.* Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* **434**, 525–529 (2005).

[150] Van Kaer, L. NKT cells: T lymphocytes with innate effector functions. *Current opinion in immunology* **19**, 354–364 (2007).

[151] Bendelac, A., Savage, P. B. & Teyton, L. The biology of NKT cells. *Annu. Rev. Immunol.* **25**, 297–336 (2007).

[152] Ziketara, N. *et al.* Critical role for miR-181a/b-1 in agonist selection of invariant natural killer T cells. *Proceedings of the National Academy of Sciences* **110**, 7407–7412 (2013).

[153] Sandrock, I. *et al.* MicroRNA-181a/b-1 is not required for innate γδ NKT effector cell development. *PLoS One* **10**, e0145010 (2015).

[154] Dweep, H. & Gretz, N. miRWalk2. 0: a comprehensive atlas of microRNA-target interactions. *Nature methods* **12**, 697–697 (2015).

[155] Ding, J., Li, X. & Hu, H. TarPmiR: a new approach for microRNA target site prediction. *Bioinformatics* **32**, 2768–2775 (2016).

[156] Agarwal, V., Bell, G. W., Nam, J.-W. & Bartel, D. P. Predicting effective microRNA target sites in mammalian mRNAs. *elife* **4**, e05005 (2015).

[157] Wang, X. miRDB: a microRNA target prediction and functional annotation database with a wiki interface. *Rna* **14**, 1012–1017 (2008).

[158] Chou, C.-H. *et al.* miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions. *Nucleic acids research* **46**, D296–D302 (2018).

[159] Altin, J. G. & Sloan, E. K. The role of CD45 and CD45-associated molecules in T cell activation. *Immunology and cell biology* **75**, 430–445 (1997).

[160] Trebak, M. & Kinet, J.-P. Calcium signalling in T cells. *Nature Reviews Immunology* **19**, 154–169 (2019).

[161] Trickett, A. & Kwan, Y. L. T cell stimulation and expansion using anti-CD3/CD28 beads. *Journal of immunological methods* **275**, 251–255 (2003).

[162] Shaywitz, A. J. & Greenberg, M. E. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annual review of biochemistry* **68**, 821–861 (1999).

[163] Maekawa, Y. *et al.* Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nature immunology* **9**, 1140–1147 (2008).

[164] Liu, Y. *et al.* microRNA-181a is involved in insulin-like growth factor-1-mediated regulation of the transcription factor CREB1. *Journal of neurochemistry* **126**, 771–780 (2013).

[165] Rodriguez-Ortiz, C. J. *et al.* miR-181a negatively modulates synaptic plasticity in hippocampal cultures and its inhibition rescues memory deficits in a mouse model of alzheimer's disease. *Aging Cell* **19**, e13118 (2020).

[166] Yano, S., Ghosh, P., Kusaba, H., Buchholz, M. & Longo, D. L. Effect of promoter methylation on the regulation of IFN-γ gene during in vitro differentiation of human peripheral blood T cells into a Th2 population. *The Journal of Immunology* **171**, 2510–2516 (2003).

[167] Pasquinelli, V. *et al.* IFN-γ production during active tuberculosis is regulated by mechanisms that involve IL-17, SLAM, and CREB. *The Journal of infectious diseases* **199**, 661–665 (2009).

[168] Chahrour, M. *et al.* MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science* **320**, 1224–1229 (2008).

[169] Jiang, S. *et al.* MeCP2 reinforces STAT3 signaling and the generation of effector CD4+ t cells by promoting miR-124–mediated suppression of SOCS5. *Science signaling* **7**, ra25–ra25 (2014).

[170] Szklarczyk, D. *et al.* STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic acids research* **47**, D607–D613 (2019).

[171] Zhang, M. *et al.* miR-181a increases FoxO1 acetylation and promotes granulosa cell apoptosis via SIRT1 downregulation. *Cell death & disease* **8**, e3088–e3088 (2017).

[172] Feng, X., Zhang, C., Yang, Y., Hou, D. & Zhu, A. Role of miR-181a in the process of apoptosis of multiple malignant tumors: A literature review. *Adv Clin Exp Med* **27**, 263–270 (2018).

[173] Ribot, J. C., Lopes, N. & Silva-Santos, B. γδ T cells in tissue physiology and surveillance. *Nature Reviews Immunology* **21**, 221–232 (2021).

[174] Ge, S. *et al.* MiR-181a, a new regulator of TGF-β signaling, can promote cell migration and proliferation in gastric cancer. *Investigational new drugs* **37**, 923–934 (2019).

[175] Li, Y. *et al.* miR-181a-5p inhibits cancer cell migration and angiogenesis via downregulation of matrix metalloproteinase-14. *Cancer research* **75**, 2674–2685 (2015).

[176] Rupaimoole, R. & Slack, F. J. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nature reviews Drug discovery* **16**, 203–222 (2017).

6

Appendix A: Bioinformatical Search

Table 6.1: Output of the mRNA targets of miR-181a for mouse, obtained using miR-Walk 3.0 [154]. The columns represent, in order of appearance: the reference sequence ID of the mRNA (refseqid); its gene symbol (genesymbol); duplex information at the predicted binding site (duplex), where '&' serves as a separator, '.' represents unpaired bases, ',' represents weakly paired bases, '—' represents strongly paired bases without preference, '{}' represents weak up/downstream paired bases, and '()' represents strong up/downstream paired bases; the predicted start site (start); the predicted ending site (end); the binding probability (bindingp); the predicted binding energy (energy); and the predicted accessibility (accessibility).

refseqid	genesymbol	duplex	start	end	bindingp	energy	accessibility
NM_009183	St8sia4	....((((.(((((..(((((((&)))))))..)))).).)))).	1553	1574	0.8076923076923077	-16.6	0.01292335
NM_001286728	Glcci1	..((((.((((((((((((((((&))))..)))))))).)))))..)))).	3381	3407	0.8076923076923077	-18.0	0.0406916
NM_001286729	Glcci1	..((((.((((((((((((((((&))))..)))))))).)))))..)))).	3378	3404	0.8076923076923077	-18.0	0.0406916
NM_010554	Il1a	((((((((((((((.......&)))))).).)))))))))).	1416	1435	0.823076923076923	-19.6	0.02265538
NM_001310698	Lrig2	...(((((((((.((((((((((&)))))))))).....))))))))).	4004	4029	0.8461538461538461	-21.0	0.001148305
NM_001311113	Syncrip	(((((((...((((((((((...&))))).....))))))))))))).	3094	3117	0.8461538461538461	-17.4	0.002903177
NM_001347345	Foxp1	(((((((...(((((((.(((.&)))))))))))...))).)))).	7188	7211	0.8461538461538461	-18.8	0.001582967
NM_001347601	Fbxl3	((((((((((...(((..(((((&)))))...)))....)))))))))).	1848	1874	0.8461538461538461	-18.9	0.01439157
NM_001358204	Atp6v1a	.((((((..((((((((((((.&))))))))))))).....)))))).	3901	3933	0.8461538461538461	-20.5	0.0003511441
NM_001360341	Fbxl3	((((((((((...(((..(((((&)))))...)))....)))))))))).	1992	2018	0.8461538461538461	-18.9	0.01439157
NM_007508	Atp6v1a	.((((((..((((((((((((.&))))))))))))).....)))))).	3887	3919	0.8461538461538461	-20.5	0.0003511441
NM_007936	Epha4	..(((((....((((((((((&))))))))).....))....))))).	4744	4780	0.8461538461538461	-18.2	0.003401774
NM_007936	Epha4	..(((((..((((((((((((.&))))))..)))))))))))).	4705	4726	0.8461538461538461	-18.1	0.001453846
NM_009723	Atp2b2	.(((((((..((((((((((.((&)).)))))))).....))).))))))).	4552	4586	0.8461538461538461	-19.1	0.007020849
NM_013625	Pafah1b1	..(((.(((.((((.(((((((&))))))).).)))))).))).	3481	3503	0.8461538461538461	-19.8	4.683667e-06
NM_013864	Ndrg2	.(((((((((((((((((....&))))))))).....)))).)))).	1739	1767	0.8461538461538461	-17.7	0.0002214723
NM_015822	Fbxl3	((((((((((...(((..(((((&)))))...)))....)))))))))).	2127	2153	0.8461538461538461	-18.9	0.01439157
NM_018823	Nfat5	.(((..((((((((((((....&))))))))).....)))))..))).	5237	5259	0.8461538461538461	-20.4	0.0002222591
NM_019460	Sfmbt1	...(((((..((((((.(((((((&))))).)))))))..))))).	4242	4263	0.8461538461538461	-18.6	0.0001188009
NM_021716	Fign	.....(((((((.(((((((((&))))))))).).....)))))))).	2878	2911	0.8461538461538461	-21.2	0.02244244
NM_021716	Fign	.((((((.(((.((((((.((((.&))))).)))))))).)))))).	7131	7152	0.8461538461538461	-19.3	0.008841147
NM_001035246	Trpm3	..(((((.((((((((((.(((&))).)))..)))))))).))))).	1387	1411	0.8461538461538461	-19.1	0.000398975
NM_001036684	Atp2b2	.(((((((..((((((((((.((&)).)))))))).....))).))))))).	4526	4560	0.8461538461538461	-19.1	0.007020849
NM_001042591	Arrdc3	....(((.((.(((((((....&))))))).))))).	3342	3357	0.8461538461538461	-17.2	0.01826225
NM_001145952	Lpp	.((((((...((((.(((.(((.&))).))).)))).....)))))).	11234	11257	0.8461538461538461	-19.4	0.0001313326
NM_001145954	Lpp	.((((((...((((.(((.(((.&))).))).)))).....)))))).	10648	10671	0.8461538461538461	-19.4	0.0001313326
NM_001145959	Ndrg2	.(((((((((((((((((....&))))))))).....)))).)))).	1697	1725	0.8461538461538461	-17.7	0.0002214723
NM_001166532	Sfmbt1	...(((((..((((((.(((((((&))))).)))))))..))))).	4385	4406	0.8461538461538461	-18.6	0.0001188009
NM_001195065	Phactr2	.(((((((.((.((.(((((((.&))))))).)).)).))))))).	3181	3203	0.8461538461538461	-20.5	0.01217756
NM_001197321	Foxp1	(((((((...(((((((.(((.&))))))))))...))).)))).	6643	6666	0.8461538461538461	-18.8	0.001582967

Table 6.1 continued from previous page

refseqid	genesymbol	duplex	start	end	bindingp	energy	accessibility
NM_080555	Plpp3	((((((.((((((((((((.(((&))).)))))..))))..)))).))))))).	1621	1648	0.8461538461538461	-19.7	0.0004683093
NM_144825	Taok1	...((((((((((((.(((((.&))))).)))..........)))))))))).	7657	7691	0.8461538461538461	-17.9	0.02174909
NM_172694	Megf9	(((((((..(((.(((((((((.&))))))).))))))...))))))).	2218	2242	0.8461538461538461	-19.7	0.03912552
NM_172839	Ccnj	((((.(((((((((..(((((((&)).)))))))))).)))))..)))).	2223	2247	0.8461538461538461	-17.9	0.0004692568
NM_173185	Csnk1g1	..((((..(((((.(((((((.((&)))))))).))).))....)))).	3648	3672	0.8461538461538461	-18.1	2.897662e-05
NM_175380	Gpd1l	.........(((((.(((((((&))))))))))))).	2431	2444	0.8461538461538461	-17.3	8.304741e-05
NM_177034	Apba1	....((((((((((((((((.&))))))..........))))))))))).	3291	3336	0.8461538461538461	-24.2	0.02524664
NM_177412	Tmcc1	........(((((((((((((..&)))))))))))))).	5099	5114	0.8461538461538461	-18.7	0.0001780862
NM_178665	Lpp	.((((((...((((.(((.(((.&))).))).))))....)))))).	11441	11464	0.8461538461538461	-19.4	0.0001313326
XM_006531192	Nfat5	.(((..(((((((((((....&)))))))))...)))))..))).	5213	5235	0.8461538461538461	-20.4	0.0002222591
NM_021716	Fign	(((.((((.((((((((((((.&))))))))))))).)..)))))).	4351	4375	0.8615384615384615	-19.7	0.0003305914
NM_001316664	Kcnk10	......(((.((((.(((.((((&))))))))))).))).	3014	3030	0.9230769230769231	-17.0	0.01967472
NM_001361386	Fam171a1	..........(((((((((.((((&))))))))))))).	2393	2406	0.9230769230769231	-21.3	0.009104876
NM_007936	Epha4	..((((((.(((((((.(((((.&))))).)))))))..............)))))).	3748	3782	0.9230769230769231	-18.1	0.0001738378
NM_009622	Adcy1	..(((((((.((((..(((((.&)))))..)))).))))))))).	8814	8835	0.9230769230769231	-20.9	0.001166652
NM_009677	Ap1g1	.((((((.((.((((.(((((.(&).)))))))).)).)..)))))).	4102	4125	0.9230769230769231	-20.3	3.686516e-06
NM_010264	Nr6a1	.((((((((((....((((((.&))))))....)))))))))).	3715	3737	0.9230769230769231	-20.7	0.01335227
NM_011603	Tbpl1	.(((((.((((((((((.((((&)))).)))))))).)))).....))))).	1211	1239	0.9230769230769231	-18.6	0.0282161
NM_013625	Pafah1b1	....(((..((.((((((((((&)))))))))).))..))).	3978	3998	0.9230769230769231	-21.2	0.001766985
NM_018871	Ywhag	((((((((((((((.((.((((.&.)))).)).))..))...)))))))))).	1923	1973	0.9230769230769231	-19.7	8.148955e-05
NM_019796	Syncrip	(((((((...((((((((((...&))))).....)))))))))))))).	3254	3277	0.9230769230769231	-17.4	0.002903177
NM_001081164	Otud4	..(((((((.((((((((((..&)))))).........)))))))))))).	4868	4896	0.9230769230769231	-19.7	6.359521e-05
NM_001142411	Zfp937	..(((((((.((((.(.((((((&))))))).).))))..))))))).	4410	4433	0.9230769230769231	-22.0	0.0002510769
NM_001145952	Lpp	...(((((....((((((((((&))))))))))...))))).	12564	12584	0.9230769230769231	-18.8	0.005351851
NM_001145954	Lpp	...(((((....((((((((((&))))))))))...))))).	11978	11998	0.9230769230769231	-18.8	0.005351851
NM_001159548	Nr6a1	.((((((((((....((((((.&))))))....)))))))))).	3407	3429	0.9230769230769231	-20.7	0.01335227
NM_001242380	Slc4a10	.....(((((((.((..(((((&)))))..))..)))))))).	3542	3562	0.9230769230769231	-18.4	0.0004235394
NM_001256033	Otud4	..(((((((.((((((((((..&)))))).........)))))))))))).	4871	4899	0.9230769230769231	-19.7	6.359521e-05
NM_001284328	Syncrip	(((((((...((((((((((...&))))).....)))))))))))))).	3251	3274	0.9230769230769231	-17.4	0.002903177
NM_001289921	Rorb	.((((((...((((((((((.&))))))).))))).....)))))).	1664	1690	0.9230769230769231	-19.6	0.01391943
NM_001301211	Ap1g1	.((((((.((.((((.(((((.(&).)))))))).)).)..)))))).	4093	4116	0.9230769230769231	-20.3	3.686516e-06
NM_001302886	Prdm4	.(((((((..(((...((((((.&))))))))))))))))).	3677	3694	0.9230769230769231	-18.4	0.001260564
NM_029083	Ddit4	.(((((((..((.(((.(((((.&))))).))).)).))))))).	1368	1389	0.9230769230769231	-18.5	0.0008196442
NM_033552	Slc4a10	.....(((((((.((..(((((&)))))..))..)))))))).	3452	3472	0.9230769230769231	-18.4	0.0004235394
NM_175445	Rassf2	..........(((((.(((((((&)))))))...))))).	4243	4259	0.9230769230769231	-18.3	0.02037049

Table 6.1 continued from previous page

refseqid	genesymbol	duplex	start	end	bindingp	energy	accessibility
NM_176832	Spire1	((((((((((.(((((((..((.&))..)))))))).)).)))))))).	1998	2022	0.9230769230769231	-20.1	0.004468192
NM_178164	Ptbp3	((((((((((((((((((.((((&)))).)))..)))))))).....)))))))).	4803	4835	0.9230769230769231	-18.6	0.004959274
NM_178665	Lpp	...(((((....((((((((((&)))))))))...))))).	12771	12791	0.9230769230769231	-18.8	0.005351851
NM_194061	D630045J12Rik	.(((((((((((((((((((((&))).)))))))))).....))).))))))).	6941	6981	0.9230769230769231	-22.1	0.01152482
XM_006495650	Creb1	.((((((.((...((((((((((&)))))))))..)))))))).	5472	5493	0.9230769230769231	-19.6	0.01594264
XM_006506310	Tmcc1	.......(((((((((((((..&)))))))))))))).	4577	4592	0.9230769230769231	-18.7	0.0001780862
XM_006526206	Spire1	((((((((((.(((((((..((.&))..)))))))).)).)))))))).	4662	4686	0.9230769230769231	-20.1	0.004468192
NM_001347067	Fam171a1	.(((((((((.(((((((((((&)))))))).....)))))).)...)))))))).	3959	4005	1.0	-23.7	0.0004735473
NM_001361386	Fam171a1	.(((((((((.(((((((((((&)))))))).....)))))).)...)))))))).	3864	3910	1.0	-23.7	0.0004735473
NM_001362490	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7864	7906	1.0	-26.0	0.002845327
NM_001362496	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7866	7908	1.0	-26.0	0.002845327
NM_001362500	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7747	7789	1.0	-26.0	0.002845327
NM_001362506	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7738	7780	1.0	-26.0	0.002845327
NM_001362507	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7744	7786	1.0	-26.0	0.002845327
NM_007936	Epha4	.(((((((((((((.((((...&)))).)))))))))...))))).	3156	3179	1.0	-19.6	4.038488e-05
NM_009952	Creb1	.((((((.((...((((((((((&)))))))))..)))))))).	5600	5621	1.0	-19.6	0.01594264
NM_010264	Nr6a1	.(((((((((.(((((.(((((.&.)))))))))).))))))))).	1877	1919	1.0	-25.2	8.290121e-05
NM_010788	Mecp2	....((((((((((((((((.&))))))))))).))).....)))))).	1920	1948	1.0	-21.3	0.0003167305
NM_011595	Timp3	.((((((..((((((((((.(((&))).)))))))))).)..)))))).	2327	2351	1.0	-27.7	0.0001494753
NM_013625	Pafah1b1	.((((((...(((((((((((.&)))))))).....)))))....)))))).	5337	5384	1.0	-18.4	0.05104301
NM_001035239	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7832	7874	1.0	-26.0	0.002845327
NM_001035240	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7832	7874	1.0	-26.0	0.002845327
NM_001035241	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7802	7844	1.0	-26.0	0.002845327
NM_001035242	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7796	7838	1.0	-26.0	0.002845327
NM_001035243	Trpm3	(((.(((((((((((.(((((.&.)))))))))))))))..))).	7766	7808	1.0	-26.0	0.002845327
NM_001037726	Creb1	.((((((.((...((((((((((&)))))))))..)))))))).	5436	5457	1.0	-19.6	0.01594264
NM_001081161	Fam171a1	.(((((((((.(((((((((((&)))))))).....)))))).)...)))))))).	3979	4025	1.0	-23.7	0.0004735473
NM_001081979	Mecp2	....((((((((((((((((.&))))))))))).))).....)))))).	1796	1824	1.0	-21.3	0.0003167305
NM_001159548	Nr6a1	.(((((((((.(((((.(((((.&.)))))))))).))))))))).	1569	1611	1.0	-25.2	8.290121e-05
NM_001310434	Fam171a1	.(((((((((.(((((((((((&)))))))).....)))))).)...)))))))).	3750	3796	1.0	-23.7	0.0004735473
NM_030732	Tbl1xr1	....(((((((((((..(((.&))).)))))))))))).	6138	6157	1.0	-20.4	0.0034089
NM_030750	Sgpp1	.(((((((((((((((.....&))).)))..))))))))))).	2087	2108	1.0	-17.9	0.03064228
NM_133828	Creb1	.((((((.((...((((((((((&)))))))))..)))))))).	5558	5579	1.0	-19.6	0.01594264
NM_144904	Ptbp3	.(((((((((((....((((..&))))...))))))))))).	4102	4122	1.0	-19.2	0.008751383
NM_146095	Rorb	.((((((...(((((((((((.&)))))))..)))))).....)))))).	1611	1637	1.0	-19.6	0.01391943

Table 6.1 continued from previous page

refseqid	genesymbol	duplex	start	end	bindingp	energy	accessibility
NM_176832	Spire1	.(((((((.(((((((.....&.)))))))).))))))).	4529	4559	1.0	-22.8	0.001229498
NM_177341	Trpm3	(((.((((((((((((.(((((.&.)))))))))))))))))..))).	6174	6216	1.0	-26.0	0.002845327
NM_178164	Ptbp3	.((((((((((((....((((..&))))...)))))))))))).	4077	4097	1.0	-19.2	0.008751383
XM_006526206	Spire1	.(((((((.(((((((.....&.)))))))).))))))).	7193	7223	1.0	-22.8	0.001229498

Table 6.2: List of either IFN-γ-associated or IL-17-associated γδ T cell genes, compiled from sequecing studies from the host lab and a bibliography search [49, 53, 79].

IFN-γ-associated genes	IL-17-associated genes
Bcl2a1a	Ahr
Ccl5	Batf
Ccr2	Blk
Ccr9	Ccr6
Cd27	Csf2
Egr2	Dkk3
Egr3	Etv5
Eomes	Hes1
Gzma	Il17a
Gzmb	Il17f
Id2	Il1r1
Id3	Il22
Ifitm1	Il23r
Ifng	Il17r
Il18r1	Irf4
Il2rb	Itk
Il4	Lef1
Klrb1c	Maf
Klrk1	Relb
Lef1	Rora
Lilr4b	Rorc
Lilrb4a	Scart1
Ly6c2	Scart2
Nfkbiz	Slamf1
Nod1	Stat5
Notch1	Sox13
Nr4a2	Sox4
Nr4a3	Tcf1
S1pr1	Zap70
Tbx21	
Tcf12	
Tcf7	
Tcf3	
Tnf	
Tnfrs18	
Zbtb16	