The role of microRNA-181a in the development and differentiation of pro-inflammatory γδ T cell subsets

Marta Silva
marta.g.silva@tecnico.ulisboa.pt

Instituto Superior Técnico, Lisboa, Portugal

July 2021

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

1. Introduction

γδ T cells are T lymphocytes that express the γ and δ chains in their T cell receptor (TCR), and that respond to pathogens and tumors by rapidly providing the pro-inflammatory cytokines interleukin-17A (IL-17) and/or interferon-γ (IFN-γ). The acquisition of effector function by γδ T cells has been demonstrated to depend on TCR signaling strength, with strong TCR signals leading to a commitment toward an γδIFN fate, and weak TCR signals promoting the development of γδ17 cells, during a process of "thymic pre-programming" that starts in embryonic development [1].

1.1. γδ T cell biogenesis

Mammal Hemapoietic Stem Cells (HSCs) residing in the fetal liver and the bone marrow (BM) seed the thymus, where they give rise to different types of mature T cells. 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 evidence pointing that commitment to the γδ lineage requires that DN cells receive stronger TCR signaling [2, 3].

TCRγδ diversity is generated through a V(D)J recombination process characterized by an ordered arrangement that leads to a timed production of specific γδ subsets that populate different tissues, thus defining developmental waves [4, 5].

The repertoire of antigens that γδ T cells are able to recognize is more limited than that of αβ T cells [6, 7], but they have a crucial role in immune responses against tumors, viruses and other pathogens [8]. In mice, most γδ T cells acquire their effector functions in the thymus, upon which they move mostly to non-lymphoid tissues, [1, 6, 9], where they represent a significant portion of all T cells present ($\sim$ 50%) [10]. Moreover, since γδ T cells mature in the thymus and certain populations home to specific tissues already endowed with a pro-inflammatory phenotype, that makes them very fast in initiating effector functions at those sites, having a key role in the early phase of immune responses. Therefore, 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 [9, 11, 12].

1.2. Pro-inflammatory γδ T cell subsets

The effector functions of γδ T cells are mostly associated with the production of two cytokines: IFN-γ and IL-17A (simplified to IL-17). γδIFN cells are associated with the defense against viral and intracellular bacterial infec-

tions [13], as well as against cancer cells [8], and γδ17 cells are important in the early stages of defense against several extracellular pathogens [14]. However, overproduction of either of these cytokines can lead to several complications, including an exacerbated reaction to severe malaria [15], and autoimmunity [14], respectively.

Pro-inflammatory cytokine production by γδ T cell subsets associates, in different developmental waves, to certain Vγ-expressing populations [14, 16–19]. In early development (E13 – E16), the first wave is marked by IFN-γ production associated to Vγ5$^+$ cells, which populate the epidermis of the skin. The following waves are marked by IL-17 production, first by Vγ6$^+$ cells (E14 – E20), that populate several epithelial tissues, and then by Vγ4$^+$ cells (E16 onwards), which home mostly to lymphoid tissues, like the spleen or lymph nodes. The final waves are characterized by the IFN-γ producers, Vγ1$^+$ and Vγ7$^+$ cells (E18 onwards), who home to lymphoid tissues and the intestine, respectively. Vγ1-expressing cells are mainly IFN-γ producers, but they are known to be able to express small amounts of IL-17 as well [20].

1.2.1 Cell Surface Markers

An important cell surface marker to identify and analyse γδ T cells subsets is CD27 [18]. Using CD27 expression, γδ T cells can be categorized into two groups: CD27$^-$, also referred to as γδ27$^-$, which are mainly IL-17 producers, but can be induced to co-express both IL-17 and IFN-γ under inflammatory conditions [21], and CD27$^+$, or γδ27$^+$, who are exclusively IFN-γ producers and represent the most significant proportion of all γδ T cells ($\sim 70 - 90\%$) in peripheral lymphoid organs under steady-state.

Another seminal work revealed that the earliest γδ T cell populations in thymus progress from being immature CD25$^+$CD24$^+$, to CD25$^-$CD24$^+$, and finally to mature CD25$^-$CD24$^-$ [22]. The mature CD24$^-$ γδ T cells progress from an uncommitted stage, in which they are CD44$^-$CD45RB$^-$ to either an IL-17 pathway, in which they upregulate CD44 to become CD44^{hi}CD45RB$^-$, or to an IFN-γ pathway, in which commitment is characterized by the expression of CD45RB. The IFN-γ pathway goes from the CD44$^-$CD45RB$^+$ stage to the CD44$^+$CD45RB$^+$ one, acquiring a more effector phenotype with the upregulation of CD44.

1.2.2 TCR signaling strength and γδ T cell effector fate

There is strong evidence pointing to a strong TCR signal inducing an IFN-γ effector potential [19, 23, 24], likely mediated by the ERK/MAPK pathway [22]. For example, a significant increase in the number of CD44$^+$CD45RB$^+$ cells (IFN-γ producers) at the expense of γδ17 cells was observed when adding anti-TCRδ to E15 thymic lobes, thus boosting TCR signal strength. This affected not only absolute cell numbers, but also cytokine production, in a dose-dependent manner. However, 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 [19, 22, 25], some studies contradict this notion, since, for instance, γδ17 cells express markers related to strong TCR engagement [26, 27].

1.3. MicroRNAs

MicroRNAs (miRNAs or miRs) are small noncoding RNA molecules, around 22 nucleotides long which, in mammals, are involved in repressing mRNA expression [28].

In mammals, miRNAs are normally generated from primary transcripts (pri-miRNAs) produced by RNA polymerase II. Within the primary transcript, the miRNA-encoding portions form imperfect stem-loop hairpin structures. Then, the Drosha type III RNase and 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. There, they are further processed by another protein complex containing the Dicer type III RNase; the miRNA is thus cleaved to its mature size. 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 usually 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) [29].

In mammals, repression of a target mRNA by a given miRNA through sequence complementarity can be induced by the miRISC complex via deadenylation, translational repression, decapping and 5'-3' degradation [30].

1.3.1 MicroRNAs in T cells

miRNAs have been widely studied in T cells, and they can either promote or suppress their activation, differentiation and/or function [31]. Supporting this is the fact that 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 [32, 33]. Furthermore, the miRNA landscape dramatically changes upon T cell activation [34]. While several miRNAs have been involved in the regulation of αβ T cell functions, very little is known about the role of miRNAs in γδ T cell development and differentiation, with only miR-146a having been described to be functionally relevant for γδ T cell plasticity and differentiation [35].

1.3.2 miR-181

The miRNA-181 family is a highly conserved family of microRNAs in vertebrates [36]. 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, namely in the bone marrow, the spleen, the thymus, the retina, and the brain [37–39], and it has been implicated in several biological processes, ranging from myoblast differentiation to various types of cancer [40,41], and T cell development [42]. miR-181a is highly expressed at the DP stage of thymocyte development [42], and it has been described as an important regulator of T cell sensitivity to antigens and in promoting T cell activation [43, 44].

In the seminal work by Li et al., the authors disclosed the effect of miR-181a as a rheostat of TCR sensitivity [43]. In this study, miR-181a expression was found to be dynamically regulated during T cell maturation, being high in the double negative (DN)1-3 and double positive (DP) stages, but dropping in DN4 and immature single positive (ISP) CD4/8. Also, miR-181a overexpression led to a significant increase in intracellular calcium upon TCR stimulation, indicating that its expression amplifies TCR-mediated T cell activation. miR-181a was found to cause the downregulation of multiple inhibitory phosphatases, which include the tyrosine phosphatases: SHP-2 and PTPN22, and also the DUSP-5 and -6 from the ERK pathway [45]. 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 [46, 47]. Additional studies show that ablation of miR-181a leads to dampening of TCR and ERK signaling, thus increasing the threshold of positive selection, without causing an autoimmune phenotype [48], and that miR-181a is responsible for sustained ERK phosphorylation in Th17 cells, which leads them to having a lower threshold of activation to antigens when compared to other Th cells [49].

1.3.3 Preliminary results

The host lab performed preliminary experiments with double reporter mice, 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. GFP$^-$eYFP$^-$ (DN), GFP+ and eYFP$^+$ subsets of γδ T cells were sorted from pLNs and subjected to small RNA-seq. This analysis revealed 103 differentially expressed miRNAs, between the IL-17$^+$ and IFN-γ$^+$ γδ T cell populations, with miR-181a being slightly overexpressed in IFN-γ$^+$eYFP$^+$ cells compared to IL-17A$^+$GFP$^+$ cells, but particularly overexpressed in the DN subset, when compared to the effector subsets.

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). 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.

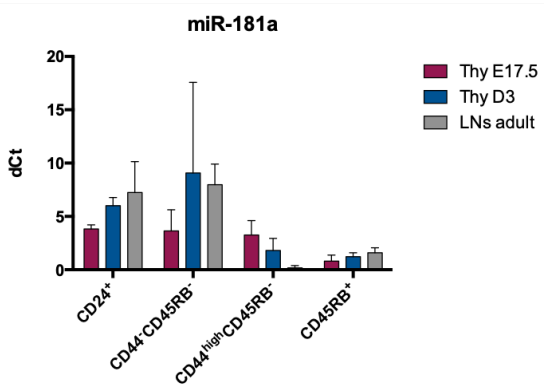

Figure 1: miR-181a expression in CD24$^+$, CD44$^-$CD45RB$^-$, CD44^{hi}CD45RB$^-$ and CD45RB$^+$ subsets of the embryonic E17.5 thymus, of the neonatal D3 thymus and of the peripheral lymph nodes of adult mice.

2. Materials and methods

2.1. Mice

We used miR-181a/b-1 KO mice (B6.*Mirc14*$^{tm1.1Ankr}$), which were described in [50]. 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.

2.1.1 Genotyping

We performed DNA extraction from a piece of the tail of each mice using the Xpert directXtract PCR kit (GRiSP) and then performed the PCR reaction using the Xpert Fast DNA Polymerase in a MyCycler Thermal Cycler (Bio-Rad).

2.1.2 Dissection

pLNs (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.

2.2. 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), CD25 (PC61), CD27 (LG.7F9), CD44 (IM7), 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.

2.3. Cell Preparation

Cell suspensions were obtained from thymus, spleen or pLNs. 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).

2.4. 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).

2.5. 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).

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

γδ27$^+$ and γδ27$^-$ cells were FACS-sorted from the pLNs of adult mice 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).

2.7. Target Gene Prediction

Predicted and validated targets for mmu-miR-181a were determined using miRWalk 3.0 [51]. 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 [52], and theTargetscan and the miRDB platforms [53,54]. The validated targets included in miRWalk 3.0 are from miRTarBase [55].

2.8. 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.

3. Results

3.1. *Ex Vivo* Analysis of Adult Lymphoid Organs

In order to test if miR-181a impacts on γδ T cell biology, we analysed *ex vivo* γδ T cells from the pLNs of miR-181a-deficient mice versus miR-181a-sufficient littermate controls by flow cytometry.

γδ T cell frequency and numbers were higher in miR-181a$^{-/-}$ mice when compared to the WT littermate controls (Figure 2(a)), but intracellular staining of the proliferation marker KI-67 on γδ T cells was similar between WT and KO animals (data not shown). Regarding TCR diversity, miR-181a$^{-/-}$ mice showed higher proportion of Vγ1$^+$ and a lower proportion of Vγ4$^+$ γδ T cells when compared to WT mice, with similar frequencies of the vestigial Vγ5$^+$ population (in pLN) (Figure 2(c)). In agreement with the association between effector markers and Vγ chain expression, miR-181a$^{-/-}$ showed a decreased frequency of the IL-17-committed CD44^{hi}CD45RB$^-$ cells, and an increased frequency and/or total cell count of IFN-γ committed CD45RB$^+$ subsets, with the major difference being observed in the less effector population of the two (CD44$^-$CD45RB$^+$). These differences did not translate into cytokine production by γδ T cells, 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) (Figure 2(b)).

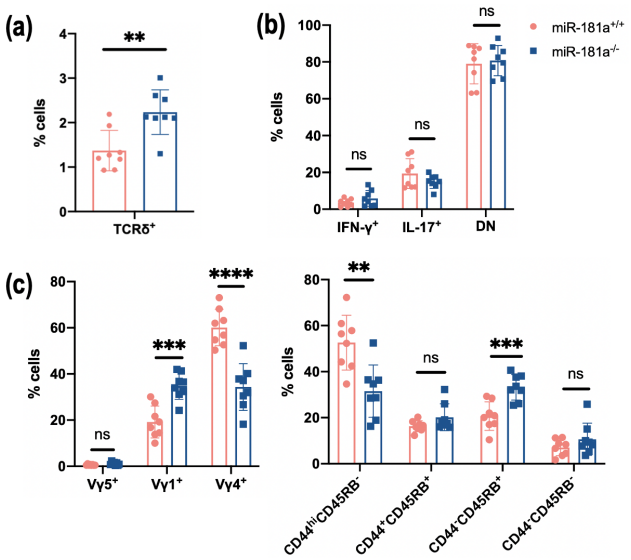

Figure 2: Main results of the *ex vivo* analysis of adult γδ T cells from the pLNs. **(a)** Frequency of γδ T cells (Live$^+$CD45$^+$CD3$^+$TCRδ$^+$) in the pLNs of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice. **(b)** Cell frequency of IFN-γ$^+$, IL-17$^+$ and DN cells ((CD3$^+$TCRδ$^+$CD24$^-$), in the pLNs of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice. **(c)** Cell frequency of Vγ1 and Vγ4 usage, and of four distinct γδ T cell subsets – CD44^{hi}CD45RB$^-$, CD44$^+$CD45RB$^+$, CD44$^-$CD45RB$^+$ and CD44$^-$CD45RB$^-$ – (CD3$^+$TCRδ$^+$CD24$^-$) in the pLNs of 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 $\leqslant$ 0.01, *** $\leqslant$ 0.001, **** $\leqslant$ 0.0001 (Mann-Whitney two-tailed test).

Considering these data, we thus hypothesized that miR-181a plays a role in γδ T cell biology in adult mice, restraining not only the frequency and total number of γδ T cells, but also the propotion of its two pro-inflammatory subsets, shifting its balance towards the IL-17 pathway. miR-181 is also associated with a decreased Vγ1/Vγ4 chain usage ratio.

3.2. *Ex Vivo* Analysis of E17.5 Thymus

Since we could not attribute the differences observed between peripheral γδ T cells of miR-181a$^{-/-}$ and miR-181$^{+/+}$ mice to differential proliferation, and both Vγ chain usage and effector fate are, in its majority, established in the thymus, we decided to analyse thymi at embryonic day E17.5 by flow cytometry, as the development of some γδ T cell subsets is restrained to this embryonic time window.

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 number (data not shown), and we did not see a difference in terms of Ki-67^{+} γδ T cells (data not shown). miR-181a$^{-/-}$ mice had slightly more Vγ1^{+} cells in terms of frequency, but not in terms of total cell numbers, and Vγ4 expression was similar between the two groups (Figure 3(a)). WT and miR-181a-KO mice had similar cell frequencies and/or numbers for the γδIFN populations, but IFN-γ MFI was higher in KO cells (Figure 3(c)). On the other hand, the frequency of the CD44^{hi}CD45RB^{-} γδ17 population is 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 [8]. A decreased frequency in terms of IL-17^{+} cells can also be seen in KO mice, although that does not reflect in terms of total cell number or IL-17 MFI (Figure 3(b)).

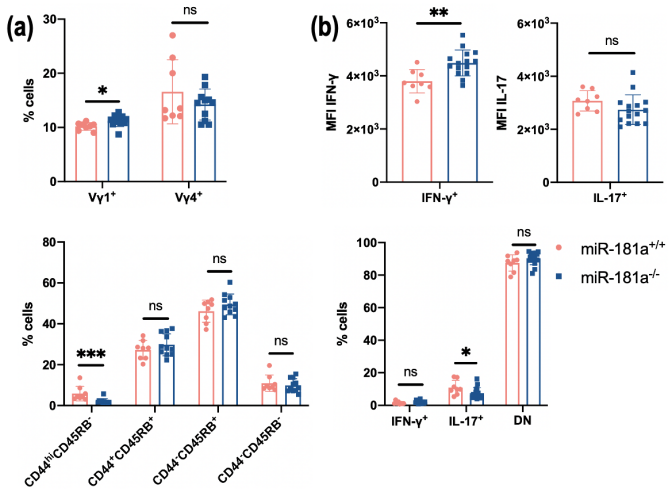

Figure 3: Main results of the *ex vivo* analysis of thymic E17.5 γδ T cells. **(a)** Cell frequency of thymic Vγ1 and Vγ4 usage, and of four distinct γδ T cell subsets – CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} – (CD3^{+}TCRδ^{+}CD24^{-}) of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ E17.5 mice. **(b)** Cell frequency and/or MFI of IFN-γ-, IL-17-expressing, and DN 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 ⩽ 0.05, ** ⩽ 0.01, *** ⩽ 0.001 (Mann-Whitney two-tailed test).

Based on the analysis of E17.5 thymi, we hypothesized that miR-181a promotes the differentiation of γδIL-17 cells, while conversely diminishing IFN-γ production by γδIFN cells.

3.3. *Ex Vivo* Analysis of D3 Thymus

Having observed some significant differences in the fetal thymus, we asked if miR-181a also had an impact in cells developing at the neonatal phase. We thus analysed the day 3 (D3) thymus of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice *ex vivo* and by flow cytometry.

We observed once again a higher frequency of γδ T cells, but not of total cell numbers, in mice lacking miR-181a, while the frequency of proliferating γδ T cells was similar between WT and KO mice (data not shown). miR-181a$^{-/-}$ mice showed increased frequencies of Vγ5^{+} and Vγ1^{+} cells, compared to miR-181a$^{+/+}$ mice, while there were no differences in terms of Vγ4 expression between the two groups (Figure 4(b)). The cell frequency of the CD44^{hi}CD45RB^{-} γδ17 population was significantly decreased in KO mice; in contrast, the CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} γδIFN subsets were significantly increased. CD44^{+}CD45RB^{+} subset does not differ much between WT and KO mice. Noticeably, these observations were not present at the E17.5 stage, but some of them remained consistent moving into lymphoid organs of adult mice; namely, the frequency of the CD44^{hi}CD45RB^{-} and CD44^{-}CD45RB^{+} populations. However, after acquiring effector functions in the thymus, most γδ T cells move to non-lymphoid tissues [8], so it is possible that some of the cells that exhibit the aforementioned differences at D3 migrated to tissues that were not analysed in this work. Also, miR-181a$^{-/-}$ mice have more IFN-γ^{+} and IL-17^{+} cells than miR-181a$^{+/+}$ mice, and a higher MFI for both cytokines (Figure 4(a)). Differences in MFI were found only in the CD44^{-}CD45RB^{-} subset, in that KO mice have a significantly higher MFI for both cytokines in this subset (Figure 4(c)).

3.4. Calcium Flux Assay

In order to test if miR-181a affects TCR signaling in γδ T cells through the mobilization of calcium, we isolated cells from the pLNs of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice and conducted a calcium flux assay, as described in the methods section of this document.

A slight, but non-significant, increase in calcium flux was observed in miR-181a$^{-/-}$ mice, in comparison to the WT littermate controls, for both αβ and γδ T cells (see Figure 5(a)). The CD44^{hi}CD45RB^{-} population of both miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice behaved similarly. Indeed, it has been shown that only the IFN-γ "pathway" is significantly responsive to calcium flux upon TCR signaling [27], 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, with the most pronounced changes occurring for the CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets (see Figure 5(b)). These data hint at 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, but this hypothesis requires further investigation.

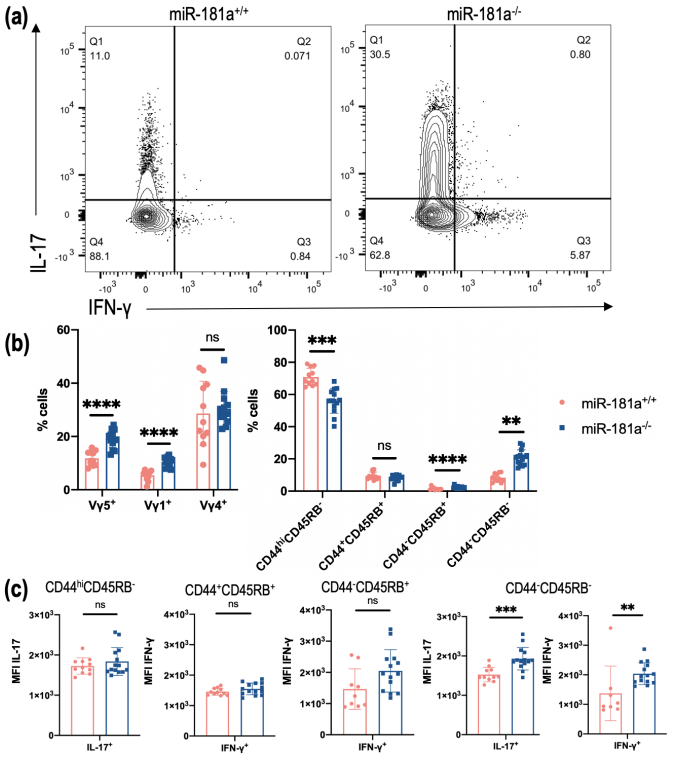

Figure 4: Main results of the *ex vivo* analysis of thymic D3 cells. **(a)** Flow cytometry analysis of intracellular IFN-γ and IL-17 expression of thymic cells of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice. **(b)** Cell frequency of thymic Vγ1 and Vγ4 usage, and of four distinct γδ T cell subsets – CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} – (CD3^{+}TCRδ^{+}CD24^{-}) of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ D3 mice. **(c)** MFI of IFN-γ- and/or IL-17-expressing thymic cells, of 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 ** ⩽ 0.01, *** ⩽ 0.001, **** ⩽ 0.0001 (Mann-Whitney two-tailed test).

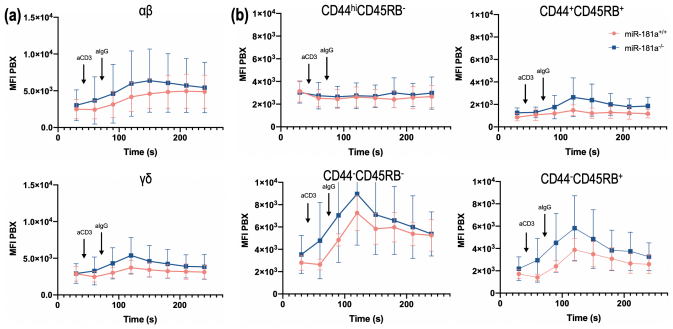

Figure 5: Main results of the calcium flux assay in T cell subsets. **(a)** Plots showing the MFI of PBX (calcium signal enhancer) throughout time, in seconds, for αβ and γδ T cells and for miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice. **(b)** Plots showing the MFI of PBX throughout time, in seconds, for the CD44^{hi}CD45RB^{-}, CD44^{+}CD45RB^{+}, CD44^{-}CD45RB^{+} and CD44^{-}CD45RB^{-} subsets of γδ T cells, of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ mice. The data shown is from two individual experiments, each consisting of n = 2.

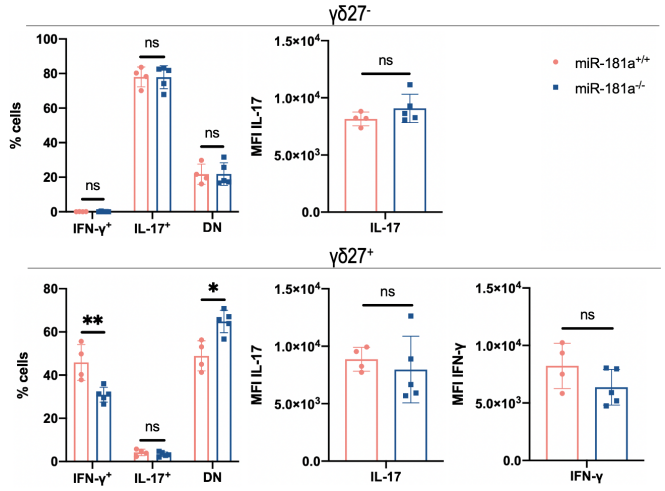

Figure 6: Frequency and MFI of IFN-γ- and IL-17-expressing γδ27^{-} and γδ27^{+} T cells, of miR-181a$^{+/+}$ and miR-181a$^{-/-}$ adult mice, after αCD3/αCD28 overnight stimulation. The data shown is from two independent experiments, one consisting of n = 3 (miR-181a$^{+/+}$) and another of n = 2 (miR-181a$^{-/-}$). Each symbol represents an individual mouse. * P ⩽ 0.05, ** ⩽ 0.01 (Mann-Whitney two-tailed test).

3.5. 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 cultured the γδ27^{-} and γδ27^{+} subsets in αCD3/28 overnight, activating them in a way that partially mimics short-term TCR stimulation [56]. Some of the samples were analyzed for proliferation after a total of 48h in culture.

The γδ27^{+} subset of miR-181a$^{-/-}$ mice showed a decreased frequency of IFN-γ^{+} cells upon *in vitro* TCR stimulation (Figure 6(a)). The γδ27^{-} subset displayed similar cell frequencies between groups. The comparison of miR-181a$^{+/+}$ with miR-181a$^{-/-}$ mice revealed similar MFIs of IFN-γ and IL-17 in both subsets. Thus, miR-181a appears to promote the frequency of γδ27^{+} cells producing IFN-γ, while the amount of cytokine produced by γδ27^{+} cells remained unaffected.

CellTrace Violet (CTV) produces a highly stable fluorescent signal that becomes diluted upon every cell division, thus allowing for the identification of several generations of cells. CTV expression indicated that there were no differences in terms of cell proliferation between miR-181a-KO mice and their WT littermate control counterparts in neither γδ27^{-}, nor γδ27^{+} cells (data not shown).

3.6. Bioinformatical Target Search

There are several validated targets of miR-181a affecting T cell differentiation already described in the literature. In αβ T cells, TCR-mediated T cell activation occurs through the repression of expression of multiple inhibitory tyrosine phosphatases: the SHP-2 and PTPN22, and also DUSP-5 and -6 from the ERK pathway [43, 45]. In Th17 cells, miR-181a is responsible for sustained ERK phosphorylation, which renders them a lower threshold of activation to antigens when compared to other Th cells, and *Id3* was pinpointed to be activated upon this sustained phosphorylation and to negatively regulate *Rorc* and, consequently, *Il17* [49]. Therefore, in order to better understand the molecular/biological basis of the effects of miR-181a on γδ T cell differentiation described throughout this work, 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 by setting the threshold for the binding

probability to 0.5 and searching for possible targets within the 3' UTR region [51]. 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 [18, 57, 58]. As such, we built two STRING networks, having set the strength of interaction to a minimum of 0.7, and having hidden disconnected nodes (data not shown).

In the IFN-γ-related STRING network, 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 [59, 60]. CREB activation was also shown to play an important role in governing the IL-2 and IFN-γ production by Th1 cells [61], thus promoting Th1 responses, with IL-17 inhibiting CREB-mediated IFN-γ production [62]. 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 [63]. 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 [64].

The IL-17-related STRING network only exhibited genes that were promotors of the IL-17 machinery. 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.

4. Discussion

miRNAs have been shown to act as post-transcriptional regulators of effector function of various T cell subsets, including the $CD4^+$ T cell proinflammatory counterparts of γδ T cell subsets, Th1 and Th17 cells. To date, however, only miR-146a has been thoroughly studied in γδ T cells [35], where it was identified to act as an IFN-γ inhibitor and to restrict γδ T cell functional plasticity *in vitro* and *in vivo*. As for miRNA-181a, it has been shown to be a regulator of TCR sensitivity to antigens and to promote conventional T cell activation [43], 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 [65], 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. 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.

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 a decreased frequency of $CD44^{hi}CD45RB^+$ cells in miR-181a-deficient 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. However, when moving towards the neonatal and adult stages, we observed not only a decreased frequency of IL-17-associated γδ T cell subsets, but also an increased frequency of IFN-γ-associated γδ T cell subsets in KO animals. 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 likely promotes the differentiation of immature cells of the γδ27$^+$ subset into effector IFN-γ$^+$ cells.

We know that miR-181a acts as a rheostat of TCR signaling strength through the modulation of several phosphatases [43]. In γδ T cells, a strong TCR signal is associated first with γδ T cell lineage commitment (over the αβ fate), and then, in terms of effector function acquisition, with IFN-γ commitment over IL-17 [1]. 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 sustained into adulthood. At E17.5 and D3, miR-181a expression favors the γδ17 subset, while also seemingly suppressing the IFNγδ populations. One study described 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 [49]. This mechanism was evident for Th17 cells 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, 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 to 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" uncommitted (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 [43, 44], 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 translates to the periphery. Then, in adults, miR-181a may mediate TCR signalling strength, affecting the differentiation of DN cells into γδIFN T cells. This mechanism is illustrated in Figure 7.

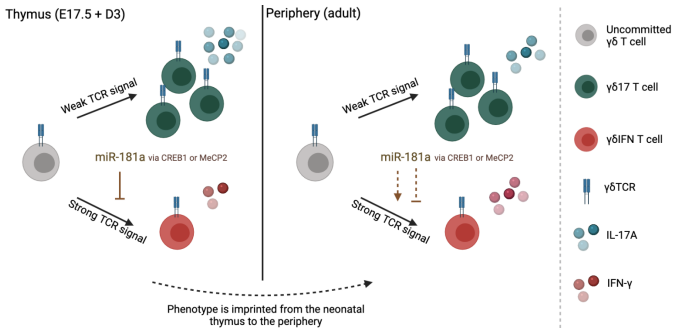

Figure 7: Proposed mechanism of the role of miR-181a in γδ T cell development. We propose that miR-181a expression prevents strong TCR signals in the fetal and neonatal thymus, thus promoting γδ17 differentiation. The phenotype observed in the neonatal thymus is imprinted into the periphery. Then, in 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.

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 [8], 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 [66].

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, to determine whether the increased differentiation into IFN-γ^{+} cells within the γδ27^{+} subset upon short-term TCR stimulation would still occur after a long-term TCR stimulation. It could also be interesting to assess how miR-181a controls the balance of the two pro-inflammatory cytokines produced by γδ T cells upon *in vivo* challenge, since adult peripheral γδ T cells are dependent on stimuli to exert their functions [8]. To do so, several 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.

Combining our model with the results from our bioinformatical search allowed us to identify a few interesting possible targets. Our two most promising mRNA targets are CREB1 and MeCP2, that promote IFN-γ production [60, 61, 63]; 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 [43, 49]. 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 if 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 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.

5. Acknowledgmens

This document was written and made publicly available as an institutional academic requirement and as a part of the evaluation of the MSc thesis in Biomedical Engineering of the author at Instituto Superior Técnico (IST). The experimental work presented in this thesis was performed at Instituto de Medicina Molecular, during the period September 2020 – March 2021, under the supervision of Prof. Dr. Anita Q. Gomes and Prof. Dr. Bruno Silva-Santos, and co-supervided at IST by Prof. Dr. Cláudia Lobato da Silva.

I acknowledge the supervisors and my tutor, Daniel Inácio, for the opportunity to work in this project and for all their guidance. I would also like to thank my family, Manel

and my friends for all their love and support.

References

[1] Nital Sumaria, Stefania Martin, and Daniel J Pennington. Developmental origins of murine γδ T-cell subsets. *Immunology*, 156(4):299–304, 2019.

[2] Maria Ciofani and Juan Carlos Zúñiga-Pflücker. Determining γδ versus αβ T cell development. *Nature Reviews Immunology*, 10(9):657–663, 2010.

[3] Mariëlle C Haks, Juliette M Lefebvre, Jens Peter H Lauritsen, Michael Carleton, Michele Rhodes, Toru Miyazaki, Dietmar J Kappes, and David L Wiest. Attenuation of γδTCR signaling efficiently diverts thymocytes to the αβ lineage. *Immunity*, 22(5):595–606, 2005.

[4] Wendy L Havran and James P Allison. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature*, 335(6189):443–445, 1988.

[5] SR Carding, S Kyes, EJ Jenkinson, R Kingston, K Bottomly, JJ Owen, and AC Hayday. Developmentally regulated fetal thymic and extrathymic T-cell receptor gamma delta gene expression. *Genes & development*, 4(8):1304–1315, 1990.

[6] Simon R Carding and Paul J Egan. γδ T cells: functional plasticity and heterogeneity. *Nature reviews immunology*, 2(5):336–345, 2002.

[7] Benjamin E Willcox and Carrie R Willcox. γδ TCR ligands: the quest to solve a 500-million-year-old mystery. *Nature immunology*, 20(2):121–128, 2019.

[8] Bruno Silva-Santos, Karine Serre, and Håkan Norell. γδ T cells in cancer. *Nature reviews immunology*, 15(11):683–691, 2015.

[9] Gina J Fiala, Anita Q Gomes, and Bruno Silva-Santos. From thymus to periphery: Molecular basis of effector γδ-T cell differentiation. *Immunological Reviews*, 298(1):47–60, 2020.

[10] Jan D Haas, Sarina Ravens, Sandra Düber, Inga Sandrock, Linda Oberdörfer, Elham Kashani, Vijaykumar Chennupati, Lisa Föhse, Ronald Naumann, Siegfried Weiss, et al. Development of interleukin-17-producing γδ T cells is restricted to a functional embryonic wave. *Immunity*, 37(1):48–59, 2012.

[11] Adrian C Hayday. γδ T cell update: adaptate orchestrators of immune surveillance. *The Journal of Immunology*, 203(2):311–320, 2019.

[12] Julie C Ribot, Miguel Chaves-Ferreira, Francisco d'Orey, Mélanie Wencker, Natacha Gonçalves-Sousa, Jérémie Decalf, João P Simas, Adrian C Hayday, and Bruno Silva-Santos. 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(11):6421–6425, 2010.

[13] Kenji Hiromatsu, Yasunobu Yoshikai, Gero Matsuzaki, Shouichi Ohga, Katsumi Muramori, Ken Matsumoto, Jeffrey A Bluestone, and Kikuo Nomoto. A protective role of gamma/delta t cells in primary infection with *Listeria monocytogenes* in mice. *The Journal of experimental medicine*, 175(1):49–56, 1992.

[14] Pedro H Papotto, Julie C Ribot, and Bruno Silva-Santos. IL-17+ γδ T cells as kick-starters of inflammation. *Nature immunology*, 18(6):604, 2017.

[15] Prasanna Jagannathan, Charlie C Kim, Bryan Greenhouse, Felistas Nankya, Katherine Bowen, Ijeoma Eccles-James, Mary K Muhindo, Emmanuel Arinaitwe, Jordan W Tappero, Moses R Kamya, et al. Loss and dysfunction of vδ2+ γδ T cells are associated with clinical tolerance to malaria. *Science translational medicine*, 6(251):251ra117–251ra117, 2014.

[16] Immo Prinz, Bruno Silva-Santos, and Daniel J Pennington. Functional development of γδ T cells. *European journal of immunology*, 43(8):1988–1994, 2013.

[17] Rebecca L O'Brien and Willi K Born. γδ T cell subsets: a link between TCR and function? In *Seminars in immunology*, volume 22, pages 193–198. Elsevier, 2010.

[18] Julie C Ribot, Ana Debarros, Dick John Pang, Joana F Neves, Victor Peperzak, Scott J Roberts, Michael Girardi, Jannie Borst, Adrian C Hayday, Daniel J Pennington, et al. CD27 is a thymic determinant of the balance between interferon-γ- and interleukin 17–producing γδ T cell subsets. *Nature immunology*, 10(4):427–436, 2009.

[19] Miguel Muñoz-Ruiz, Julie C Ribot, Ana R Grosso, Natacha Gonçalves-Sousa, Ana Pamplona, Daniel J Pennington, José R Regueiro, Edgar Fernández-Malavé, and Bruno Silva-Santos. TCR signal strength controls thymic differentiation of discrete proinflammatory γδ T cell subsets. *Nature immunology*, 17(6):721–727, 2016.

[20] Luigina Romani, Francesca Fallarino, Antonella De Luca, Claudia Montagnoli, Carmen D'Angelo, Teresa Zelante, Carmine Vacca, Francesco Bistoni, Maria C Fioretti, Ursula Grohmann, et al. Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature*, 451(7175):211–215, 2008.

[21] Nina Schmolka, Karine Serre, Ana R Grosso, Margarida Rei, Daniel J Pennington, Anita Q Gomes, and Bruno Silva-Santos. Epigenetic and transcriptional signatures of stable versus plastic differentiation of proinflammatory γδ T cell subsets. *Nature immunology*, 14(10):1093–1100, 2013.

[22] Nital Sumaria, Capucine L Grandjean, Bruno Silva-Santos, and Daniel J Pennington. Strong TCRγδ signaling prohibits thymic development of IL-17A-secreting γδ T cells. *Cell reports*, 19(12):2469–2476, 2017.

[23] Kirk DC Jensen, Xiaoqin Su, Sunny Shin, Luke Li, Sawsan Youssef, Sho Yamasaki, Lawrence Steinman, Takashi Saito, Richard M Locksley, Mark M Davis, 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(1):90–100, 2008.

[24] Pablo Pereira, Claire Berthault, Odile Burlen-Defranoux, and Laurent Boucontet. Critical role of TCR specificity in the development of vγ1vδ6. 3+ innate nktγδ cells. *The Journal of Immunology*, 191(4):1716–1723, 2013.

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

[26] Francis Coffey, Sang-Yun Lee, Terkild B Buus, Jens-Peter Holst Lauritsen, Gladys W Wong, Michelle L Joachims, Linda F Thompson, Juan Carlos Zúñiga-Pflücker, Dietmar J Kappes, and David L Wiest. The TCR ligand-inducible expression of cd73 marks γδ lineage commitment and a metastable intermediate in effector specification. *Journal of Experimental Medicine*, 211(2):329–343, 2014.

[27] Melanie Wencker, Gleb Turchinovich, Rafael Di Marco Barros, Livija Deban, Anett Jandke, Andrew Cope, and Adrian C Hayday. Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. *Nature immunology*, 15(1):80–87, 2014.

[28] Victor Ambros. The functions of animal microRNAs. *Nature*, 431(7006):350–355, 2004.

[29] Scott D Boyd. Everything you wanted to know about small RNA but were afraid to ask. *Laboratory investigation*, 88(6):569–578, 2008.

[30] Stefanie Jonas and Elisa Izaurralde. Towards a molecular understanding of microRNA-mediated gene silencing. *Nature reviews genetics*, 16(7):421–433, 2015.

[31] Daniel P Inácio, Tiago Amado, Bruno Silva-Santos, and Anita Q Gomes. Control of T cell effector functions by miRNAs. *Cancer letters*, 427:63–73, 2018.

[32] Mark MW Chong, Jeffrey P Rasmussen, Alexander Y Rudensky, and Dan R Littman. The RNAseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease. *The Journal of experimental medicine*, 205(9):2005–2017, 2008.

[33] Stefan A Muljo, K Mark Ansel, Chryssa Kanellopoulou, David M Livingston, Anjana Rao, and Klaus Rajewsky. Aberrant T cell differentiation in the absence of dicer. *The Journal of experimental medicine*, 202(2):261–269, 2005.

[34] Silvia Monticelli, K Mark Ansel, Changchun Xiao, Nicholas D Socci, Anna M Krichevsky, To-Ha Thai, Nikolaus Rajewsky, Debora S Marks, Chris Sander, Klaus Rajewsky, et al. MicroRNA profiling of the murine hematopoietic system. *Genome biology*, 6(8):1–15, 2005.

[35] Nina Schmolka, Pedro H Papotto, Paula Vargas Romero, Tiago Amado, Francisco J Enguita, Ana Amorim, Ana F Rodrigues, Katrina E Gordon, Ana S Coroadinha, Mark Boldin, et al. MicroRNA-146a controls functional plasticity in γδ T cells by targeting NOD1. *Science immunology*, 3(23), 2018.

[36] Zhen Yang, Xueshuai Wan, Zhuoya Gu, Haohai Zhang, Xiaobo Yang, Lian He, Ruoyu Miao, Yang Zhong, and Haitao Zhao. Evolution of the mir-181 microRNA family. *Computers in biology and medicine*, 52:82–87, 2014.

[37] Chang-Zheng Chen, Ling Li, Harvey F Lodish, and David P Bartel. MicroRNAs modulate hematopoietic lineage differentiation. *science*, 303(5654):83–86, 2004.

[38] David G Ryan, Michelle Oliveira-Fernandes, Robert M Lavker, et al. MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity. *Mol Vis*, 12(134-135):1175–1184, 2006.

[39] Eric A Miska, Ezequiel Alvarez-Saavedra, Matthew Townsend, Akira Yoshii, Nenad Šestan, Pasko Rakic, Martha Constantine-Paton, and H Robert Horvitz. Microarray analysis of microRNA expression in the developing mammalian brain. *Genome biology*, 5(9):1–13, 2004.

[40] Irina Naguibneva, Maya Ameyar-Zazoua, Anna Polesskaya, Slimane Ait-Si-Ali, Reguina Groisman, Mouloud Souidi, Sylvain Cuvellier, and Annick Harel-Bellan. The microrna miR-181 targets the homeobox protein Hox-a11 during mammalian myoblast differentiation. *Nature cell biology*, 8(3):278–284, 2006.

[41] Cecilia Pop-Bica, Sebastian Pintea, Roxana Cojocneanu-Petric, Giannino Del Sal, Silvano Piazza, Zhao-Hui Wu, Alvaro J Alencar, Izidore S Lossos, Ioana Berindan-Neagoe, and George A Calin. MiR-181 family-specific behavior in different cancers: a meta-analysis view. *Cancer and Metastasis Reviews*, 37(1):17–32, 2018.

[42] Joel R Neilson, Grace XY Zheng, Christopher B Burge, and Phillip A Sharp. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes & development*, 21(5):578–589, 2007.

[43] Qi-Jing Li, Jacqueline Chau, Peter JR Ebert, Giselle Sylvester, Hyeyoung Min, Gwen Liu, Ravi Braich, Muthiah Manoharan, Juergen Soutschek, Petra Skare, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell*, 129(1):147–161, 2007.

[44] Peter JR Ebert, Shan Jiang, Jianming Xie, Qi-Jing Li, and Mark M Davis. An endogenous positively selecting peptide enhances mature T cell responses and becomes an autoantigen in the absence of microRNA miR-181a. *Nature immunology*, 10(11):1162, 2009.

[45] Mark A Lindsay. microRNAs and the immune response. *Trends in immunology*, 29(7):343–351, 2008.

[46] Guillaume Gaud, Renaud Lesourne, and Paul E Love. Regulatory mechanisms in T cell receptor signalling. *Nature Reviews Immunology*, 18(8):485–497, 2018.

[47] Jiansheng Wu, Anjali Katrekar, Lee A Honigberg, Ashley M Smith, Marion T Conn, Jie Tang, Doug Jeffery, Kyle Mortara, Jun Sampang, Steve R Williams, et al. Identification of substrates of human protein-tyrosine phosphatase PTPN22. *Journal of Biological Chemistry*, 281(16):11002–11010, 2006.

[48] Steven A Schaffert, Christina Loh, Song Wang, Christopher P Arnold, Robert C Axtell, Evan W Newell, Garry Nolan, K Mark Ansel, Mark M Davis, Lawrence Steinman, et al. mir-181a-1/b-1 modulates tolerance through opposing activities in selection and peripheral T cell function. *The Journal of Immunology*, 195(4):1470–1479, 2015.

[49] Federico Mele, Camilla Basso, Cristina Leoni, Dominik Aschenbrenner, Simone Becattini, Daniela Latorre, Antonio Lanzavecchia, Federica Sallusto, and Silvia Monticelli. ERK phosphorylation and miR-181a expression modulate activation of human memory T_H17 cells. *Nature communications*, 6(1):1–12, 2015.

[50] Natalia Ziketara, Marcin Lyszkiewicz, Katrin Witzlau, Ronald Naumann, Robert Hurwitz, Jörg Langemeier, Jens Bohne, Inga Sandrock, Matthias Ballmaier, Siegfried Weiss, 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(18):7407–7412, 2013.

[51] Harsh Dweep and Norbert Gretz. miRWalk2. 0: a comprehensive atlas of microRNA-target interactions. *Nature methods*, 12(8):697–697, 2015.

[52] Jun Ding, Xiaoman Li, and Haiyan Hu. TarPmiR: a new approach for microRNA target site prediction. *Bioinformatics*, 32(18):2768–2775, 2016.

[53] Vikram Agarwal, George W Bell, Jin-Wu Nam, and David P Bartel. Predicting effective microRNA target sites in mammalian mRNAs. *elife*, 4:e05005, 2015.

[54] Xiaowei Wang. miRDB: a microRNA target prediction and functional annotation database with a wiki interface. *Rna*, 14(6):1012–1017, 2008.

[55] Chih-Hung Chou, Sirjana Shrestha, Chi-Dung Yang, Nai-Wen Chang, Yu-Ling Lin, Kuang-Wen Liao, Wei-Chi Huang, Ting-Hsuan Sun, Siang-Jyun Tu, Wei-Hsiang Lee, et al. miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions. *Nucleic acids research*, 46(D1):D296–D302, 2018.

[56] Annette Trickett and Yiu Lam Kwan. T cell stimulation and expansion using anti-CD3/CD28 beads. *Journal of immunological methods*, 275(1-2):251–255, 2003.

[57] Nidhi Malhotra, Kavitha Narayan, Ok Hyun Cho, Katelyn E Sylvia, Catherine Yin, Heather Melichar, Mehdi Rashighi, Veronique Lefebvre, John E Harris, Leslie J Berg, et al. A network of high-mobility group box transcription factors programs innate interleukin-17 production. *Immunity*, 38(4):681–693, 2013.

[58] Maria Pokrovskii, Josip S Herman, Shruti Naik, Elisabeth Sock, Patrice Zeis, Ute Lausch, Michael Wegner, Yakup Tanriver, Dan R Littman, and Dominic Grün. Deciphering the regulatory landscape of fetal and adult γδ T-cell development at single-cell resolution. *The EMBO journal*, 39(13):e104159, 2020.

[59] Adam J Shaywitz and Michael E Greenberg. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annual review of biochemistry*, 68(1):821–861, 1999.

[60] Yoichi Maekawa, Yoshiaki Minato, Chieko Ishifune, Takeshi Kurihara, Akiko Kitamura, Hidefumi Kojima, Hideo Yagita, Mamiko Sakata-Yanagimoto, Toshiki Saito, Ichiro Taniuchi, et al. Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nature immunology*, 9(10):1140–1147, 2008.

[61] Shingo Yano, Paritosh Ghosh, Hitoshi Kusaba, Meredith Buchholz, and Dan L Longo. 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(5):2510–2516, 2003.

[62] Virginia Pasquinelli, James C Townsend, Javier O Jurado, Ivana B Alvarez, María F Quiroga, Peter F Barnes, Buka Samten, and Verónica E García. IFN-γ production during active tuberculosis is regulated by mechanisms that involve IL-17, SLAM, and CREB. *The Journal of infectious diseases*, 199(5):661–665, 2009.

[63] Maria Chahrour, Sung Yun Jung, Chad Shaw, Xiaobo Zhou, Stephen TC Wong, Jun Qin, and Huda Y Zoghbi. MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science*, 320(5880):1224–1229, 2008.

[64] Shan Jiang, Chaoran Li, Gabrielle McRae, Erik Lykken, Jose Sevilla, Si-Qi Liu, Ying Wan, and Qi-Jing Li. MeCP2 reinforces STAT3 signaling and the generation of effector CD4+ t cells by promoting miR-124–mediated suppression of SOCS5. *Science signaling*, 7(316):ra25–ra25, 2014.

[65] Inga Sandrock, Natalia Ziketara, Marcin Łyszkiewicz, Linda Oberdörfer, Katrin Witzlau, Andreas Krueger, and Immo Prinz. MicroRNA-181a/b-1 is not required for innate γδ NKT effector cell development. *PLoS One*, 10(12):e0145010, 2015.

[66] Julie C Ribot, Noëlla Lopes, and Bruno Silva-Santos. γδ T cells in tissue physiology and surveillance. *Nature Reviews Immunology*, 21(4):221–232, 2021.