Universidade de Lisboa
Faculdade de Medicina de Lisboa

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Orientador: Professor Doutor Bruno Miguel de Carvalho e Silva Santos
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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas, especialidade de Imunologia

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Resumo

Os microRNAs são pequenas moléculas de RNA, de aproximadamente 22 nucleótidos, utilizadas pelas células no controlo da sua expressão génica. Atuam ao nível pós-transcricional, destabilizando ou inibindo a tradução de moléculas de RNA mensageiro que possuam um local para a sua ligação. São elementos necessários para o funcionamento das células e a sua importância já foi demonstrada em vários sistemas e tipos celulares, tanto em animais como em plantas e fungos. Em mamíferos, eles são necessários para o normal funcionamento das células do sistema imunitário, onde já foram, por exemplo, descritos como sendo importantes na regulação da produção de citocinas. O controlo da produção de citocinas, como as citocinas pro-inflamatórias interferão gama (IFN-γ) e interleucina 17 (IL-17), é essencial para a defesa contra infecções e para a manutenção da homeostasia do hospedeiro. A produção de IFN-γ é vital para a resposta a vírus, bactérias intracelulares e células cancerígenas, e por sua vez, a produção de IL-17 é necessária no combate contra bactérias extracelulares e fungos. No entanto, a produção em excesso ou desregulada destas citocinas pode dar origem ao desenvolvimento de inflamação crónica e doenças autoimunes, tais como esclerose múltipla, diabetes tipo I, artrite reumatóide, colite ou psoríase. Diferentes células do sistema imunitário têm a capacidade de produzir estas citocinas. Numa típica resposta adaptativa contra patogénios intracelulares as maiores fontes de IFN-γ são as células T CD8$^+$, que também apresentam grande capacidade citotóxica, em conjunto com as células T CD4$^+$. No entanto, nas fases iniciais de infecção, são as células que apresentam características mais inatas, como as células T $\gamma\delta$, que se apresentam como a fonte predominante desta citocina, uma vez que têm a capacidade de a produzir prontamente quando estimuladas. Para além de IFN-γ as células T $\gamma\delta$ são também capazes de rapidamente produzir IL-17, que é tipicamente induzida por outro tipo de estímulos e produzida como resposta a infecções por bactérias extracelulares ou fungos. Dada as graves consequências que podem advir de uma expressão de citocinas descontrolada, o controlo da sua expressão tem de ser feita de uma maneira muito rigorosa pelo que a descoberta dos mecanismos de controlo assume assim uma grande importância. É neste contexto que se insere o trabalho apresentado nesta tese.

Na primeira parte desta tese, procurámos identificar microRNAs que estivessem envolvidos na regulação da expressão de IFN-γ pelas células T CD8$^+$. Começámos por demonstrar a

importância que os microRNAs têm globalmente neste contexto, mostrando que células T CD8$^+$ desprovidas de microRNAs apresentam uma maior produção de IFN-γ. Após análise dos níveis de expressão de microRNAs em células T CD8$^+$ provenientes de ratinhos repórter para o IFN-γ (com uma construção bicistrónica de IFN-γ com a proteína fluorescente YFP), identificámos 29 microRNAs diferencialmente expressos entre células produtoras de IFN-γ (YFP$^+$) e células não produtoras desta citocina (YFP$^-$). Destes 29 microRNAs selecionamos 6 para manipular experimentalmente. Verificámos que os microRNAs miR-181a-5p e o miR-451a têm a capacidade de inibir a expressão de IFN-γ em células T CD8$^+$, através da destabilização de moléculas de mRNA de genes como o *Id2*, *Akt2* e *Map2k1*. Demonstrámos de seguida que a redução dos níveis de *Id2* leva a uma diminuição da produção de IFN-γ, fenocopiando assim os efeitos da sobreexpressão do miR-181a-5p. Estas observações suportam a hipótese apresentada nesta tese de que o miR-181a-5p é capaz de reduzir a produção de IFN-γ em células T CD8$^+$, pelo menos parcialmente, através da redução do mRNA de *Id2*. Quando em baixa quantidade, o Id2 não é capaz de inibir a ligação das proteínas E2/E47 ao *locus* do gene *Tbx21* o que leva a uma inibição da transcrição deste *locus*. Sendo *Tbx21* um factor de transcrição importante para a expressão de IFN-γ em células T CD8$^+$, a diminuição da sua expressão leva diretamente a uma redução da expressão de IFN-γ. Este estudo permitiu assim a identificação de 2 microRNAs, o miR-181a-5p e o miR-451a, que contribuem para o controlo da expressão de IFN-γ por parte de células T CD8$^+$, atuando como reguladores negativos da expressão desta citocina.

Na segunda parte desta tese, caracterizámos, através de Next Generation Sequencing (NGS), o transcriptoma e o miRNoma de duas subpopulações efetoras de células T $\gamma\delta$: as produtoras de IL-17 e as produtoras de IFN-γ, isoladas a partir de ratinhos repórter para ambas a citocinas (com construções bicistrónicas com GFP e YFP respectivamente). Com base nestas experiências identificamos os genes diferencialmente expressos entre as duas subpopulações com os objetivos de aumentar o nosso conhecimento no que diz respeito à identidade celular de cada subpopulação efetora e de identificar novos microRNAs de interesse na biologia das células T $\gamma\delta$. Esta análise revelou 103 microRNAs diferencialmente expressos de entre os quais selecionámos 8 microRNAs para estudar. Através de manipulações *in vitro* verificámos que os microRNAs miR-326-3p, miR-7a-5p

e miR-1949 são capazes de limitar a expressão de IFN-*γ;* que o miR-128-3p inibe a expressão de IFN-*γ* e promove a expressão de IL-17; e que o miR-322-5p promove a co-produção de IL-17 com IFN-*γ*. Serão ainda necessárias mais experiências a fim de determinar o impacto que estes microRNAs têm *in vivo*, nomeadamente num contexto de infecção ou doença.

A análise do transcriptoma revelou, por sua vez, 7882 genes diferencialmente expressos, dos quais 4341 estavam enriquecidos na subpopulação capaz de produzir IL-17 e 3541 na subpopulação capaz de produzir IFN-*γ*. Estes dados permitiram a obtenção de uma visão global sobre a biologia dos linfócitos T *γδ*, nomeadamente sobre as vias e processos estão mais enriquecidos em cada subpopulação deste tipo de linfócitos. Foram identificadas 62 vias de sinalização intracelular mais representadas nas células produtoras de IL-17 enquanto que nas células produtoras de IFN-*γ* foram identificadas 27 vias. Entre os resultados obtidos, diferenças na capacidade de detecção de estímulos extracelulares, incluindo de moléculas tipicamente não associadas ao sistema imunitário, como neuromediadores ou receptores de hormonas, e diferenças em vias metabólicas estão entre os mais interessantes, que merecem investigação adicional ao nível funcional.

No geral, o trabalho descrito nesta tese permitiu a identificação de vários microRNAs que regulam a expressão de citocinas pró-inflamatórias em células T CD8$^+$ ou *γδ* e realçou várias diferenças entre as subpopulações efetoras de células T *γδ*, constituindo um recurso importante para a comunidade científica formular novas hipóteses sobre a identidade celular e a fisiologia destes subtipos pouco estudados de linfócitos T.

Palavras chave: Células T; Regulação de citocinas; MicroRNAs; Transcriptoma.

Summary

MicroRNAs are small RNA molecules, approximately 22 nucleotides in length, employed by cells to regulate gene expression. They act post-transcriptionally, destabilizing or inhibiting the translation of mRNA molecules that harbour a corresponding binding site. MicroRNAs are essential for cell function as their depletion in mice is embryonically lethal. MicroRNAs are required for the correct development and function of immune cells, where, in particular, they have been implicated in the control of cytokine secretion. The control of the production of cytokines, such as pro-inflammatory interferon (IFN)-γ and interleukin (IL)-17, is essential for the defence against pathogens and for homeostasis maintenance. IFN-γ production is critical for the immune response against viruses, intracellular bacteria and tumour cells whilst IL-17 is necessary in the fight against extracellular bacteria and fungi. However, an excessive or deregulated secretion of these cytokines can lead to the development of chronic inflammation and autoimmune diseases, such as multiple sclerosis, type I diabetes, rheumatoid arthritis, colitis or psoriasis.

Different cells of the immune system have the ability to produce these cytokines. In a typical adaptive response to intracellular pathogens, the major sources of IFN-γ are $CD8^+$ T cells and their $CD4^+$ (T helper type 1, Th1) counterparts, taking on from innate-like NK and $\gamma\delta$ T cells that provide most IFN-γ during the earlier stages. As for IL-17, the lymphocyte subsets that account for most of its production during immune responses to extracellular pathogens are $\gamma\delta$ T cells (at early stages) and $CD4^+$ T helper type 17 (Th17) cells. Given the importance of a regulated expression of these pro-inflammatory cytokines, the molecular mechanisms responsible for such regulation are of utmost relevance. It is within this conceptual frame that the work presented in this thesis was developed.

In the first part of this thesis, we aimed to identify microRNAs involved in the regulation of IFN-γ production by $CD8^+$ T cells. We started by demonstrating that microRNAs are globally important in this process by showing that $CD8^+$ T cells lacking microRNAs (due to genetic deficiency in the microRNA-processing enzyme Dicer) display exacerbated IFN-γ production compared to controls. By analysing the expression levels of microRNAs in $CD8^+$ T cells from a mouse strain with a fluorescent reporter for IFN-γ (bicistronic

construction of IFN-γ with the fluorescent protein YFP), we identified 29 microRNAs differentially expressed between IFN-γ producing (YFP^+) versus non-producing (YFP^-) $CD8^+$ T cells. From these, we showed in gain-of-function experiments that microRNAs miR-181a-5p and miR-451a inhibit IFN-γ expression in $CD8^+$ T cells, by targeting and destabilizing mRNA molecules of genes such as *Id2*, *Akt2* and *Map2k1*. We further showed that a reduction in *Id2* levels leads to a reduction in IFN-γ production in $CD8^+$ T cells, thus phenocopying the effects of overexpressing miR-181-5p. These observations support the hypothesis that miR-181a-5p reduces IFN-γ production in $CD8^+$ T cells by, at least partially, reducing *Id2* mRNA levels. When present in low amounts, Id2 cannot inhibit the ligation of proteins E2/E47 to the *Tbx21* locus, which leads to the transcriptional silencing of this gene, which is an important transcription factor for the expression of IFN-γ in $CD8^+$ T cells.

In the second part of this thesis, we characterized, through Next Generation Sequencing (NGS), the transcriptome and miRNome of the two main effector subpopulations of $\gamma\delta$ T cells: IL-17 producers and IFN-γ producers. These were isolated from a reporter mouse strain for both cytokines (with bicistronic constructions for both GFP and YFP respectively). We thereby identified the genes differentially expressed between the two $\gamma\delta$ T cell effector populations aiming at improving our knowledge on their cellular identity and microRNA-mediated gene regulation. Our analysis revealed 103 differentially expressed microRNAs from which we selected 8 candidates to study in more detail. *In vitro* polarization experiments showed that miR-326-3p, miR-7a-5p and miR-1949 limit IFN-γ expression; that miR-128-3p inhibits IFN-γ expression and promotes IL-17 expression; and that miR-322-5p promotes co-production IFN-γ together with IL-17 . Further work is required to determine the impact these microRNAs may have *in vivo*, namely in the context of infection or inflammatory disease.

In turn, our transcriptome analysis revealed 7882 genes differentially expressed between effector $\gamma\delta$ T cell subsets, with 4341 enriched in $IL\text{-}17^+$ cells and 3541 enriched in IFN-γ^+ cells. The data provided a global view on the biology of $\gamma\delta$ T lymphocytes, namely on the pathways and processes enriched in each of the effector subpopulations. We identified 62 pathways enriched in the IL-17 producing subpopulation and 27 in their IFN-γ producing

counterparts. Differences in the ability to integrate external cues, including some not typically associated with the immune system, such as neuronal mediators or hormone responsiveness, and metabolic differences are amongst the most interesting and novel ones, which warrant further functional investigation.

In sum, the work presented in this thesis allowed the identification of various microRNAs involved in the regulation of cytokine expression in $CD8^+$ or $\gamma\delta$ T cells; and highlighted several unanticipated differences between effector $\gamma\delta$ T cell populations, thus constituting a useful resource for the scientific community to formulate new hypotheses on the cellular identity and physiology of $\gamma\delta$ T cells.

Key words: T cells, Cytokine regulation, MicroRNAs, Transcriptome.

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Abbreviations

Ago - Argonaute
bp - Base pair
C - Celsius
CD - Cluster of differentiation
CNS - Central nervous system
CTL - Cytotoxic T lymphocyte
DETC - Dendritic epidermal $\gamma\delta$ T cell
DN - Double negative
DNA - Deoxyribonucleic acid
DR - Double reporter
EAE - Experimental autoimmune encephalomyelitis
Eomes - Eomesodermin
eYFP - Enhanced yellow fluorescent protein
FACS - Flow activated cell sorting
FC - Fold change
FDR - False discovery rate
FSC - Forward scatter
GFP - Green fluorescent protein
GO - Gene ontology
h - Hours
HCV - Hepatitis C virus
IFN - Interferon
IL - Interleukin
IRES - Internal ribosome entry site.
KEGG - Kyoto encyclopedia of genes and genomes
KO - Knock out
LNA - Locked nucleic acid
MHC - Major histocompatibility complex
min - Minutes
miR- MicroRNA
miRNA - MicroRNA
mRNA - Messenger RNA
MS - Multiple sclerosis
MuHV-4 - Murid herpesvirus-4
NCR - Natural cytotoxicity receptor
NGS - Next generation sequencing
NK - Natural killer
NKT - Natural killer T cell
nt - Nucleotide
PCA - Principal component analysis
PCR - Polymerase chain reaction
PLN - Peripheral lymph nodes
qPCR - Quantitative polymerase chain reaction
RISC - RNA-induced silencing complex
RNA - Ribonucleic acid

RNase - Ribonuclease
ROR - RAR-related orphan receptor
RT-qPCR - Reverse transcription quantitative polymerase chain reaction
Seq - Sequencing
siRNA - Small interfering RNA
SSC - Side scatter
TCR - T cell receptor
Tfh - T follicular helper cell
Th - T helper cell
Treg - Regulatory T cell
UTR - Untranslated region
WT - Wild type
YETI - IFN-γ-IRES-YFP-BGHpolyA knockin
YFP - Yellow fluorescent protein

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INTRODUCTION

1. Introduction

1.1 - Biogenesis and function of microRNAs

MicroRNAs (miRNAs) are small (~22 nt) non-coding regulatory RNAs involved in the post-transcriptional repression of messenger RNAs (mRNAs) in a sequence specific manner.

By targeting most of the mRNAs coded in the genome and by forming complex networks between them and their targets, microRNAs influence virtually every cellular, developmental and homeostatic process in animals [1]. This is well illustrated by the diverse phenotypes and pathologies caused by their absence or deregulation [2]. In fact, and even though Dicer and Drosha are also involved in other additional processes such as transcription regulation and pre-mRNA splicing [3], deletion of either one of these enzymes, essential elements in the canonical microRNA biogenesis pathway, results in early embryonic lethality in mice, showing just how important the presence of microRNAs is [3–5].

Nowadays we can find in version 22 of miRBase, a popular microRNA sequence and annotation database (http: //mirbase.org/), over 38 500 entries for hairpin precursors microRNAs from 271 organisms, including vertebrate or invertebrate animals and plants [6,7]. In the most recent version 22.1 there are 1303 annotated hairpin precursors and 2013 mature sequences for *Mus musculus*. In the human genome those numbers are 1984 and 2683 respectively. However, due to the nature of this annotation, there might be a certain degree of false negative and also false positive annotations, the latter being the most likely [8]. Stringent criteria and high-throughput sequencing data lead to more robust annotation [2].

Despite these impressive numbers the microRNA field is relatively young, not even 3 decades old. The first small regulatory RNA was discovered in the invertebrate model organism *Caenorhabditis elegans* in 1993 and was described as a short RNA, produced by the *lin-4* locus, which had the ability to repress the *lin-14* mRNA at the post-transcriptional level, thus controlling the developmental transition between larval stages

[9,10]. Later let-7, also described in *C. elegans* and also involved in a developmental transition was found to be present in a wide range of bilaterian animal species, including vertebrates, suggesting this type of post-transcriptional control was not unique to worms [11,12]. Indeed just in the following years several reports were published identifying more of these now called microRNAs and showing that some of them were highly conserved in invertebrates and vertebrates. More interestingly they also showed that these microRNAs could have diverse expression patterns both temporal and spatial (tissue) wise [13–16]. This time and tissue-specific patterns are mainly regulated transcriptionally [17].

MicroRNAs can be encoded in diverse genomic contexts and their biogenesis is a multistep process that starts in the nucleus and ends in the cytoplasm (**Figure 1.1**). They can be transcribed independently or as part of a polycistronic unit with other, usually related, microRNAs. They are found in introns and exons of non-coding transcripts in addition to introns of mRNAs [8,18,19].

In animals, canonical microRNA genes are transcribed into primary microRNAs, called pri-miRNA, by RNA polymerase II (RNA pol II) [20–22]. These longer pri-miRNA transcripts form at least one hairpin structure, where the mature microRNA sequence is embedded, that will be recognized and serve as substrate to the microprocessor complex, composed of one RNase Drosha and two DGCR8 proteins. The two RNAse III domains of Drosha are responsible for the cleavage of both strands at the base of the stem loop, which generates a 60-70 nucleotides hairpin called pre-miRNA. At this stage an alternative cleavage by Drosha will give rise to the production of isomirs (which are variant forms of a canonical microRNA) [23–27]. Exportin 5 and RAN-GTP will recognize the pre-miRNA and export it to the cytoplasm, where it will be bound and processed by Dicer [28–31]. Dicer, like Drosha, is an enzyme containing two RNase III domains and associates with its helper proteins TRBP and PACT [32,33]. Acting as a molecular ruler this complex will cleave both strands near the bottom of the terminal loop leaving a ~22nt mature microRNA duplex that is ready to be incorporated into the Argonaute (Ago) protein [34,35]. Again, an alternative cleavage by Dicer can give rise to production of isomirs [27]. Only the mature strand of the microRNA duplex will be loaded into the Argonaute's guide-channel giving rise to the RNA-induced silencing complex (RISC), whereas the other strand will be

degraded. It is thus the mature strand loaded in the Ago protein that will provide the information on which mRNAs targets are to be inhibited by RISC. Since a microRNA gene can generate two distinct strands, there are theoretically at least two possible sets of regulatory targets for each microRNA duplex that will be defined by which strand is preferentially incorporated. This targeting ability is usually defined by the microRNA nucleotides 2-8, also called extended seed region, that the RISC will rely on to recognize mRNA targets by base complementary, mainly at their 3' untranslated region (3' UTR), and silence them [36,37].

Repression by RISC can happen in two distinct ways (**Figure 1.1**). If the pairing between the microRNA and its target transcript is extensive enough, then the complex will promote the endonucleolytic cleavage of the transcript leading to its degradation [38,39]. MicroRNAs promoting this type of silencing are commonly found in plants [40]. Mechanistically, this repression is similar to what happens in the RNA interference pathway, whose discovery constituted the basis for the revolutionizing siRNA strategy that uses small double-stranded RNA molecules to slice, and thus knockdown, complementary target mRNAs and that has been widely used in research and holds therapeutic potential [41,42]. In several mammalian species, including human and mouse, very few microRNAs seem to exert their inhibition this way. Instead, a mechanism that destabilizes the mRNA or inhibits its translation and does not involve the direct cleavage of the target transcript by RISC is favoured (**Figure 1.2**) [39,43]. It requires however, that Ago recruits auxiliary proteins such as GW182 family proteins, named trinucleotide repeat-containing 6 (TNRC6) in vertebrates. TNRC6 is in turn able to interact with the cytoplasmic poly(A)-binding protein (PABPC), associated with the target mRNA's 3' UTR, and with the cytoplasmic deadenylase complexes CCR4-NOT and PAN2-PAN3. The deadenylation of the transcript's poly(A) tail, catalysed by the CCR4-NOT and PAN2-PAN3 complexes, will lead to its destabilization by decapping and 5'-to-3' degradation by exoribonucleases [44].

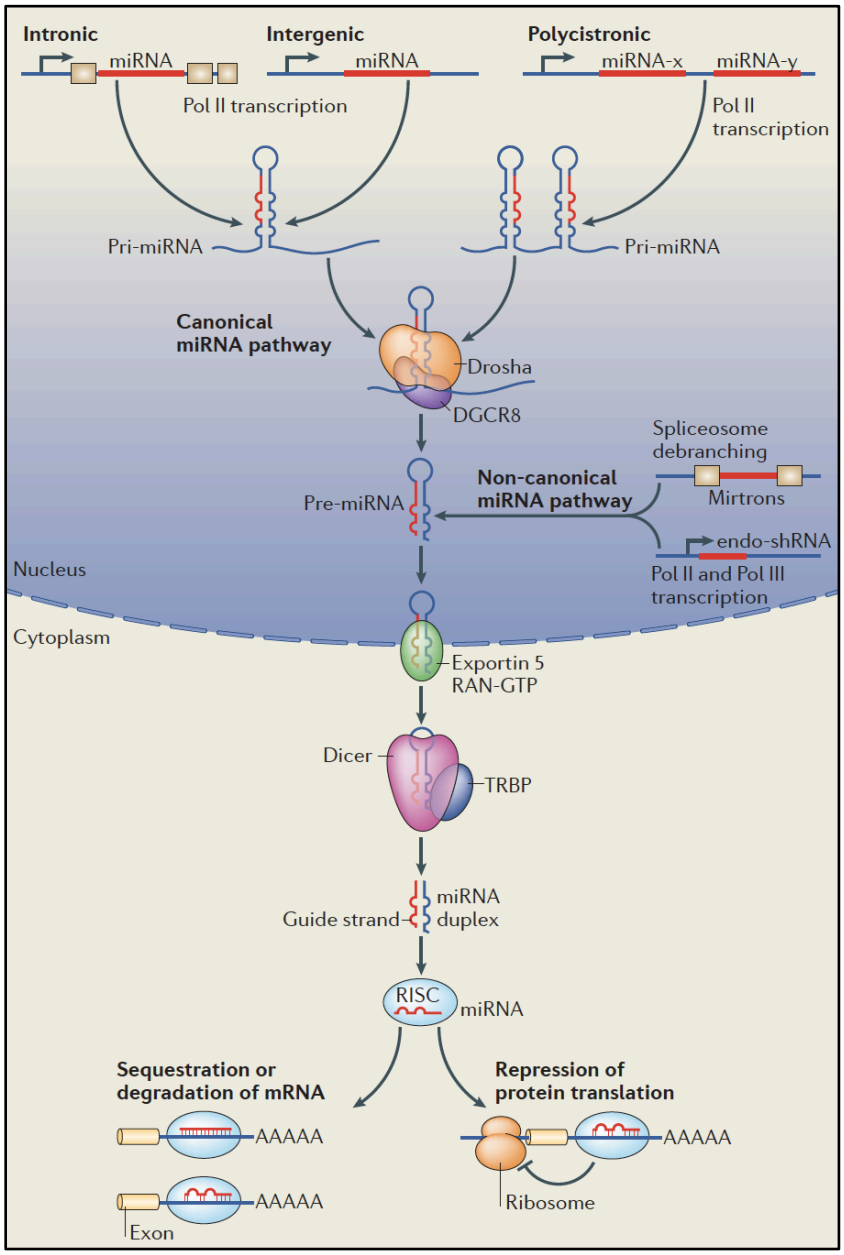


Figure 1.1 - The microRNA biogenesis pathway.
In the canonical biogenesis pathway microRNAs are transcribed as precursors RNAs by RNA polymerase II (Pol II) from intronic, intergenic or polycistronic *loci*. These primary microRNAs (pri-miRNAs) are then recognized by the Drosha/DGCR8 complex that cuts the strands on the stem of the pir-miRNA hairpin to release a trimmed ~60 nt stem loop called pre-miRNA. By the action of Exportin5 and RAN-GTP the pre-miRNAs are exported from the nucleus to the cytoplasm where they are cleaved by Dicer to generate the final ~22 nt microRNA duplex. This duplex will be loaded into Argonaute proteins where one strand will be incorporated and serve as the guide strand while the complementary strand will be expelled and degraded. The RNA-inducing silencing complex (RISC) is now assembled and will promote the destabilization or translation repression of the mRNA molecules that present target sequences recognized by the microRNA guide strand incorporated in the complex. Figure from Rottiers and Näär, 2012 [45].

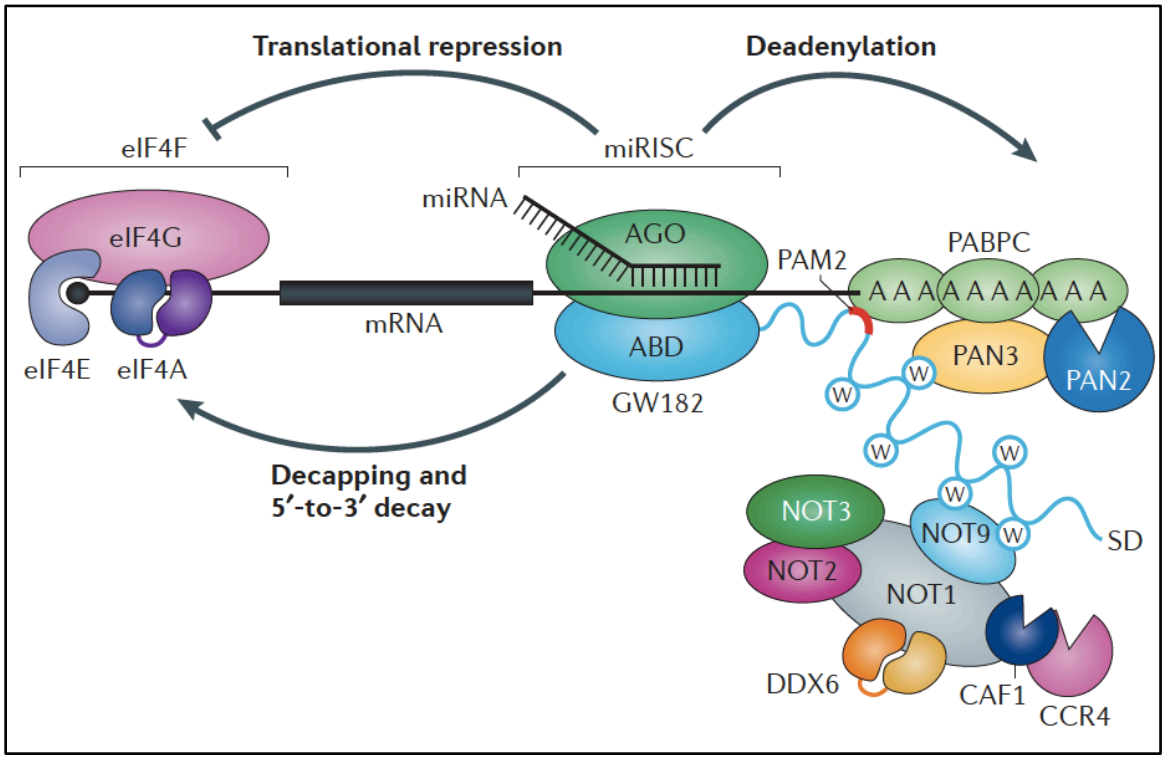


Figure 1.2 - The dominant mechanisms of microRNA-mediated gene silencing in animals. The RISC, guided by the incorporated microRNA, recognizes the mRNA target via base pairing with the partially complementary binding sites usually located in the 3' UTR of the transcript. The complex then recruits TNRC6, a GW182 family member capable of interacting with PABPC, bound to the transcript's poly(A) tail, and of recruiting the deadenylase complexes CCR4-NOT or PAN2-PAN3. Either one of the deadenylase complexes recruited catalyses the shortening of the mRNA's poly(A). This shortening will promote decapping and degradation of the mRNA by exoribonucleases except in the early embryonic stages where poly(A) shortening reduces translation initiation with little effect on mRNA stability. In addition RISC is also able to repress translation initiation through a parallel mechanism in which it interferes with the function and/or assembly of the eukaryotic initiation factor 4F (eIF4F) complex. Figure adapted from Jonas and Izaurralde, 2015 [46].

In addition, and although the exact mechanism is still not completely clear, the proteins recruited by the RISC can also inhibit the translation initiation of the microRNA target, likely by interfering with the function of the eukaryotic initiation factor 4F complex (eIF4F). DEAD box protein 6 (DDX6), recruited to the RISC by the CCR4-NOT complex and with the ability to interact with the eIF4F complex, is though to enhance the decay of the mRNA as well as its translational repression (**Figure 1.2**) [46–51].

Although they likely share common elements, these two processes can contribute differently for the microRNA-mediated gene silencing. In post-embryonic mammalian

cells, although there is a contribution from translation inhibition, RISC-induced silencing seems to be achieved mainly through mRNA destabilization, which can alone explain most of the steady state repression caused by microRNAs [52–55] .

There are some microRNA genes, called noncanonical microRNAs, whose biogenesis process differs in some steps from the one described above, since they generate their pre-miRNA or mature microRNA complex in an alternative way. For example, pre-miRNA hairpins capable of being processed by Dicer can arise from splicing of an intron. These are called "mirtrons" and their ends are defined by the spliceosome instead of by Drosha [56]. Another class of microRNAs that don't follow the canonical pathway and don't rely on Drosha is the endogenous short hairpin RNAs (shRNAs), whose hairpins arise from transcription and are ready to be processed by Dicer [57]. However once the mature microRNA duplexes are formed they will be integrated in RISC and exert their inhibitory functions just like their canonical counterparts.

1.2 - T cells and microRNA-mediated regulation of cytokine production

In humans and rodents, the immune system is composed of various cell types with specialized functions that interact with each other and with the surrounding tissues in order to protect the organism against pathogens and to maintain homeostasis.

Amongst these immune cells, T cells are notorious for their ability to discriminate self from non-self (antigen recognition) and to coordinate immune responses. T cells get their name from the fact that they develop in the thymus, where they acquire a T cell receptor (TCR) and must successfully complete a complex selection process before being allowed to leave to the periphery where they will exert their role. They can be divided in subgroups according to the surface markers they express, which reflect their different functional abilities. T cells expressing a TCR composed of a disulfide-linked heterodimer of variable α and β chains are called $\alpha\beta$ T cells. By expressing additional surface proteins such as CD4, CD8 or NK1.1 they are further classified into $CD4^+$, $CD8^+$ or Natural Killer (NKT) T cells respectively. Most $CD4^+$ $\alpha\beta$ T cells become helper T cells (Th cells) and leave the

thymus in a naïve state. They will complete their differentiation in the periphery into one of several lineages by integrating external signals and adopting specific transcriptional programs directed by each lineage's master transcription factor [58–60]. Each of these lineages is characterized by expression of their signature cytokine being IFN-γ for Th1, IL-4 for Th2, IL-9 for Th9, IL-17 for Th-17; IL-22 for Th-22 and IL-21 for Tfh **(Figure 1.3)** [61,62].

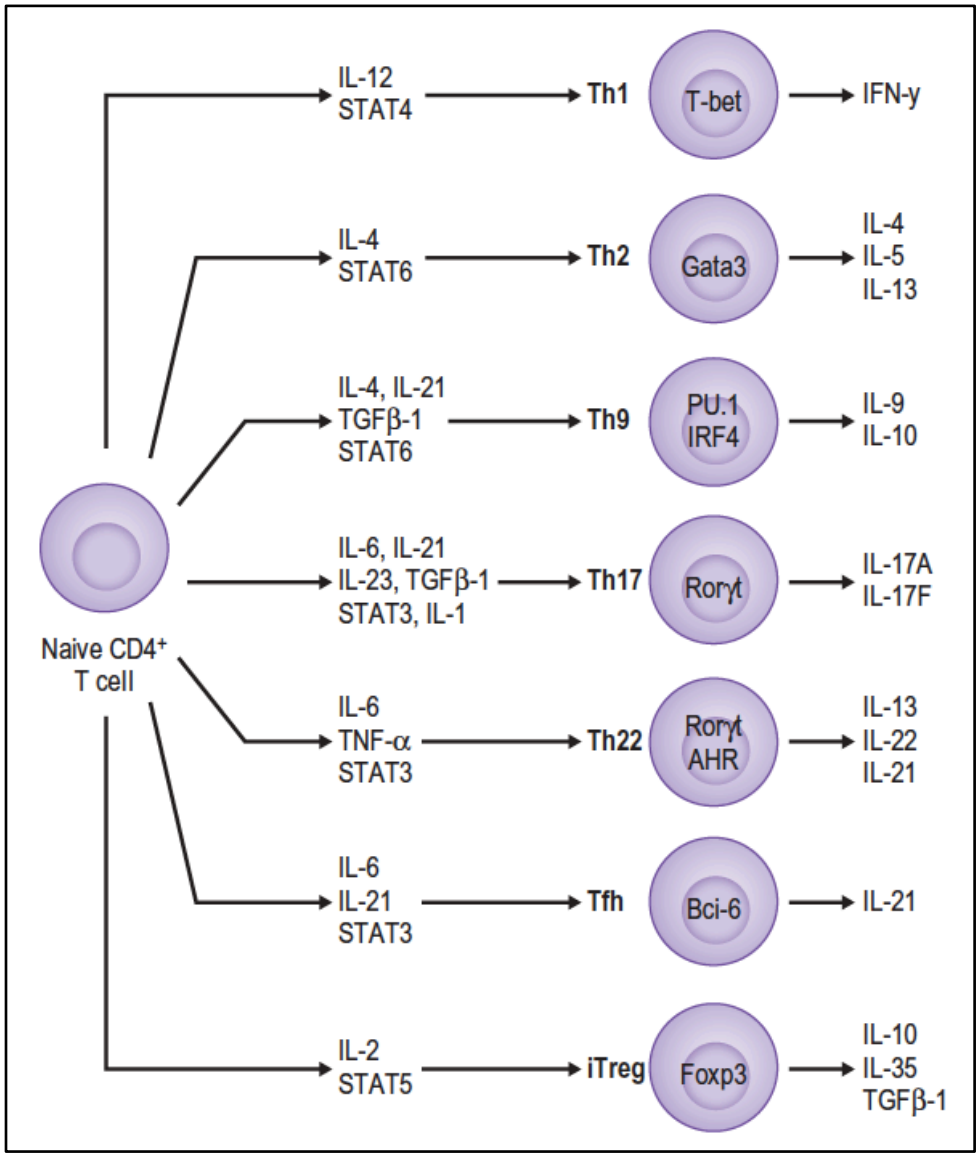


Figure 1.3 (shown on preceding page) - Differentiation of T helper cell subsets.
Upon antigen stimulation, naïve CD4+ T cells can differentiate into different types of helper cells, each of which secretes characteristic cytokines (e.g. IFN-γ for Th1). The differentiation into a particular subset is done accordingly to the signals received from the environment (e.g. IL-12 for Th1), which will trigger a signalling cascade (e.g. STAT4 for Th1) that drives the expression of the subset's master transcription factor (e.g. T-bet for Th1). Figured adapted from Rich, 2013 [63].

A small percentage of $CD4^+$ $\alpha\beta$ T cells, usually under 10%, follows an alternative path and leaves the thymus as regulatory T cells (Tregs). Characterized by expression of the master transcription factor FoxP3 and high CD25 expression, Tregs will be responsible for inhibiting immune reactions, for example by secreting IL-10, an anti-inflammatory cytokine [58]. $CD8^+$ T cells are a different type of $\alpha\beta$ T cells that, in turn, participate in immune responses by secreting several cytotoxins like perforin and granzyme but also cytokines such as IFN-γ, the main effector cytokine produced by $CD8^+$ T cells upon TCR activation. They are indispensable for an efficient host response against not only viral and intracellular bacteria infections but also against cancerous cells. NKT cells, whose distinguishing feature is the expression of natural cytotoxicity receptors (NCRs) and an invariant TCR, can also be a source of IFN-γ as well as other cytokines such as TNF-α, IL-4 or GM-CSF [63].

Less frequent in number than their $\alpha\beta$ counterparts but equal in importance, are T cells whose TCR is composed by a γ and a δ chain earning them the name of $\gamma\delta$ T cells. They are also conserved throughout evolution and found across many species but in sharp contrast to the T helper family these cells leave the thymus already committed to their functional role and, as such, are able to rapidly secrete large amounts of IL-17 or IFN-γ upon activation [63,64]. They are more frequent in tissues that are in direct contact with the exterior where they contribute to homeostasis maintenance and provide protection against distinct types of pathogens or transformed cancer cells. They show non-redundant functions with $\alpha\beta$ T cells and have been shown to play important beneficent roles in the defence of the host against infections and tumours [65,66]. Just like other T cells, they too have been shown to have pathogenic potential, being implicated in harmful inflammation and autoimmune diseases such as psoriasis, uveitis, type I diabetes and experimental autoimmune encephalomyelitis, and in certain conditions cancer [67–69].

MicroRNAs have been implicated in the development, differentiation and function of not only T cells but, if not all, many other cell types of the immune system. [37,70–75].

Although Dicer and Drosha play other roles outside the biogenesis of mature microRNAs, the importance microRNAs have in the homeostasis, differentiation and function of T

cells can be appreciated by looking at the results from experiments where these two vital enzymes were conditionally eliminated in mouse T lymphocytes.

Upon Dicer deletion, T cells were still able to form with a successfully rearranged T cell receptor but the number of total thymocytes observed was significantly reduced and defects in peripheral T cells were noticeable, namely decreased cell numbers and reduced cell proliferation [76,77]. This was at least partially due to the fact that Dicer-deficient cells proliferate slower that control cells and can more easily enter into apoptosis. Interestingly the compromised survival was mainly observed in cells from the $\alpha\beta$ lineage, while the $\gamma\delta$ lineage was found to be unscathed [76,77].

By placing $CD4^+$ naïve T cells under Th1 or Th2 promoting conditions, scientist have shown that Dicer is not required for the differentiation into IFN-γ or IL-4 expressing cells respectively. Consistently, the corresponding master transcription factors *Tbx-21* for Th1 and *Gata3* for Th2 were detected within the expected populations. At the end of the Th1 polarization protocol, there was no significant difference in the percentage of IFN-γ^+ cells between those with or without Dicer. However, Dicer deficient cells were able to reach that final number considerably sooner than cells with a functional Dicer. In non-polarizing conditions, the proportion of cells expressing IFN-γ as well as the amount of IFN-γ detected within each cell was greatly increased, as was the transcription factor *Tbx21*, in the Dicer-deficient cells when compared with their Dicer-sufficient counterparts. Under the Th2 differentiation cocktail a more peculiar phenotype was observed: even though cells lacking Dicer were just as able to produce IL-4 as control cells, the same cannot be said for their ability to repress IFN-γ expression. $Dicer^{-/-}$ cells in a Th2 inducing culture also showed higher numbers of IFN-γ^+ $IL\text{-}4^-$ cells and surprisingly of IFN-γ^+ $IL\text{-}4^+$ cells (the Th1 and Th2 program are usually described as being mutually exclusive) [77].

In an *in vivo* context, microRNAs' biogenesis in T cells is essential for homeostasis since removal of either Dicer or Drosha will result in spontaneous inflammation and autoimmunity. The absence of microRNAs in naïve cells makes them prone to develop into a strong pro-inflammatory phenotype and in Tregs hinders their correct development, number and function [78–80]. The broken balance between the effector and regulatory $CD4^+$ $\alpha\beta$ T cell population, with an increased secretion of inflammatory cytokines by the

effectors and loss of suppression ability by the regulators, is likely the reason behind the exacerbated inflammation and premature mortality observed in these animals.

Several reports suggest that, generally speaking, microRNAs prevent naïve T cells from committing prematurely to helper functions [37,74,81]. In fact, upon TCR activation, most microRNAs were shown to be downregulated as result of the proteasomal degradation of the Ago microRNA-processing proteins, and naïve T cells with lower Ago2 levels were more prone to differentiate into cytokine-producing T cells [82]. Another very interesting finding is that lymphocyte activation in associated with 3' UTR shortening [83]. Alternative polyadenylation allows mRNAs from the same *locus* to have varying lengths of their 3' UTR and since more than half of the microRNA-binding sites are downstream of the first polyadenlyation site, the usage of different 3' UTR lengths is a way to allow or avoid microRNA-mediated repression [84]. Activated and proliferating T cells seem to use these mechanisms to escape the microRNA-mediated repression in order to be able to differentiate correctly and become functionally active.

These observations, combined with the evidence that functional T cell subsets exhibit distinct microRNA profiles and that deletion of Dicer or Drosha specifically in T cells causes a profound deregulation of T cell differentiation, underlie the strong impact of microRNAs on cytokine production [37,76,77,79,82,85–87].

In fact since the early studies implying a global role for microRNAs in T cell biology, the number of scientific reports showing how important microRNAs are in regulating T cell differentiation and controlling cytokine production and how the lack of this regulation can lead to disease just keeps increasing and several individual microRNAs have been identified as regulators of cytokine expression (**Table 1.1**). This regulation can be accomplished directly, on the cytokine itself, or indirectly, for example targeting transcription factors, chromatin remodelling agents or other elements involved in the regulation of cytokine production by the distinct T cell subsets (**Table 1.1**).

We recently reviewed the impact of microRNAs on cytokines, including those regulating IL-2, IFN-γ and type 1 associated factors, IL-4 and type 2 associated factors and IL-17

and type 17 associated factors in $CD4^+$ T cells and $CD8^+$ T cells [88]. As this work addresses the role of microRNAs in $CD8^+$ and $\gamma\delta$ T cell differentiation, which produce the pro-inflammatory cytokines IFN-γ ($CD8^+$ and $\gamma\delta$ T cells) and IL-17 ($\gamma\delta$ T cells), I will briefly describe, in the next section, some examples of microRNA-mediated regulation of IFN-γ and IL-17 production by T cells.

Table 1.1 - MicroRNA, the cytokines they regulate and respective mechanisms of action.

microRNA	*Cytokine(s) modulated*	*mRNA targets & proposed mechanism*	*References*
miR-9	IL-2 [↑] IFN-γ [↑]	Blimp-1 (PRDM1) and Bcl-6 Downregulation of IL-2 and T-bet repressors	89
miR-17~92	IFN-γ [↑] IL-4 [↑] IL-5 [↑] IL-5 [↑] TNF [↓]	Pten Released repression of PI3K-Akt signalling pathway, promoting Th1 differentiation, and IFN-γ production Pten, SOCS1, and Tnfaip3 TNFα Targeting of negative regulators of Th2 differentiation	90 91,92
miR-20b	IL-17 [↓]	RORγt and STAT3 Suppression of critical Th17 differentiation factors	93
miR-21	IFN-γ [↓] IL-4 [↑]	Impacts on the balance between Th1 and Th2	94
miR-23a cluster	IFN-γ [↓]	IFN-γ CD107a	95
miR-27 and miR-128	IL-4 [↓] IL-5 [↓]	BMI1 Enhanced GATA3 degradation	96
miR-29	IFN-γ [↓]	IFN-γ, T-bet, and Eomes Suppression of essential IFN-γ differentiation factors	97–99
miR-31	IL-2 [↑]	KSR2 FoxP3 De-repression of COT/TPI2 signalling pathway, leading to enhanced NFAT activity	100
miR-125b	IFN-γ [↓] Il-2 [↓]	IFN-γ, Blimp-1, IL-2Rβ, and IL-10Rα	86
miR-132/212	IL-17 [↑]	Bcl-6 Downregulation of Th17 differentiation repressors	101
miR-146a	IFN-γ [↓] IL-17 [↓] IL-2 [↓]	IRAK1 and TRAF6 in the NF-κB and other signalling pathways	
miR-155	IL-4 [↓] IL-5 [↓] IL-13 [↑] IL-17 [↑] IL-22 [↑] IL-9 [↑]	c-Maf c-Maf promotes expression of IL-4 (and of IL-5 and IL-10 when ectopically expressed) S1pr1 c-Maf and Jarid2 c-Maf inhibits IL-22 transcription	

	IL-10 [↑] IL-17 [↑] IL-22 [↑] IFN-γ [↑] IFN-γ [↑]	Jarid deficiency causes a rearrangement of epigenetic marks that lead to the repression of IL-22, IL-10, IL-9, Atf3, T-bet, and Eomes loci. Atf3 is capable of promoting IL-17A production. Ets-1 Downregulation of a Th17 differentiation repressor SOCS1 Allows corret response to IL-2 signling and proliferation Ship1 Targeting of a negative regulator of the PI3K pathway	
miR-181c	IL-2 [↓]	IL-2	102
miR-210	IL-17 [↓]	Hif-1α Suppression of Th17 differentiation promoting factor	103
miR-301a	IL-17 [↑]	PIAS3 Downregulation of a strong inhibitor of the STAT3 pathway	104
miR-326	IL-17 [↑]	Ets-1 Downregulation of a Th17 differentiation repressor	105
miR-340	IL-4 [↓] IL-5 [↓]	IL-4 and BMI1 Enhanced GATA3 degradation	96
let-7 family	IL-17 [↓] IL-13 [↓] IL-10 [↓]	IL-23R, IL-10, and IL-13 Reduced IL-17 production	106–108

MicroRNA-mediated regulation of IFN-γ and type 1- associated factors

IFN-γ is critical for the regulation of the host immune response against viral and intracellular bacterial pathogens [109–112] and for tumour surveillance [113,114]. On the other hand excessive release of IFN-γ has been associated with chronic inflammatory or autoimmune diseases like type I diabetes, lupus, arthritis and colitis [115–117]. Over the last decade several microRNAs have been identified which regulate IFN-γ expression in $CD4^+$ (Th1) and $CD8^+$ T cells. Th1 cells differentiate in the presence of IL-12, which induces STAT-1 and STAT-4 and express the master transcription factor T-bet (encoded by *Tbx21*), whereas Eomesodermin (Eomes) is usually regarded as the master transcription factor for IFN-γ producing $CD8^+$ T cells.

MiR-29-mediated regulation of IFN-γ production is a good example of the multiple levels at which a microRNA can interfere with cytokine expression. Indeed, miR-29 targets multiple genes involved in the Th1 differentiating pathway. MiR-29 levels were found to correlate inversely with those of IFN-γ in $CD4^+$ and $CD8^+$ T cells and NK cells under Th1-polarising conditions *in vitro* and upon infection with an intracellular pathogen *in*

vivo [99]. In this work, miR-29 was shown to be a negative regulator of IFN-γ expression [99]. An independent study showed that miR-29 was induced upon T cell activation and, in the case of Th1 cells, also by IFN-γ itself via STAT1 signalling, thus establishing a negative feedback loop that prevents exaggerated expression of IFN-γ [97]. Importantly, miR-29 overexpression in DGCR8-deficient $CD4^+$ T cells was able to rescue their aberrant IFN-γ expression [98]. These studies, describing an important role for miR-29 in Th1 cell differentiation, are, however, controversial in terms of the target molecules identified. Both IFN-γ and the IFN-γ-driving transcription factors, T-bet and Eomes contain miR-29 binding sites in their 3' UTRs. One of the studies showed that miR-29 inhibits the expression of IFN-γ by direct targeting of the 3' UTR of IFN-γ mRNA, with no effect on the IFN-γ-driving transcription factors, T-bet and Eomes [99]. In the two other studies, miR-29 was described either to bind to the 3' UTR of both IFN-γ and T-bet mRNAs [97] or to bind both T-bet and Eomes 3' UTRs [98]. The reasons behind these discrepancies are still unclear.

The miR-17-92 cluster promotes the expression of IFN-γ in $CD4^+$ T cells. Deletion of this cluster in mouse $CD4^+$ T cells has been shown to reduce both the percentage of IFN-γ-producing cells and the amount of IFN-γ produced per cell [90]. As a consequence, miR-17-$92^{f/f}$ CD4-Cre^+ mice showed impaired anti-tumour response and increased tumour burden in the B16 melanoma mouse model [90]. The miR-17-92 cluster (mainly miR-19b) targets PTEN, which is a negative regulator of the PI3K-Akt signalling pathway required for Th1 differentiation and IFN-γ production and could therefore account for the observed changes [90,118]. Similarly, miR-155 promotes IFN-γ expression in mouse $CD4^+$ and $CD8^+$ T cells by repressing Ship1, another negative regulator of the PI3K signalling pathway [119].

MiR-146a, on the other hand, was found to be highly expressed in Tregs and to be vital for their ability to control IFN-γ-mediated Th1 immune responses, since the loss of miR-146a in Tregs resulted in an increased production of IFN-γ by $CD4^+$ and $CD8^+$ effector T cells whereas no changes in the production of IL-4, IL-5 or IL-17 were detected [120]. MiR-146a impacts on the suppressive function of Tregs by directly targeting STAT1 mRNA and promoting its degradation. Thus miR-146a contributes to the continuous suppression of the Th1-like features in Tregs and supports the maintenance of the Treg identity [120]. MiR-146a is also involved in Toll-like and cytokine receptors signalling by targeting IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) 3'

UTRs, two adaptor molecules downstream of NF-κB signalling [121–124]. MiR-146a is therefore a negative regulator of T cell activation and miR-146a$^{-/-}$ mice showed an increase production of several cytokines including IFN-γ, IL-17 and IL-2 [123].

The expression of miR-9 was found to be upregulated in human CD4$^+$ T cells upon activation, and was shown to increase IFN-γ production [89]. Amongst the predicted targets of miR-9 are the 3' UTRs of the *Blimp-1* and *Bcl-6* genes' transcripts. Blimp-1 has previously been implicated in the repression of IL-2, Fos and T-bet in T cells; and Bcl-6 in the repression of T-bet [125,126]. Importantly, the overexpression of miR-9 was shown to decrease Blimp-1 and Bcl-6 expression, while it augmented IFN-γ production by CD4$^+$ T cells [89].

A separate report in human T cell subsets also identified Blimp-1 and IFN-γ mRNAs as being targets of miR-125b, a signature microRNA of naïve CD4$^+$ T cells responsible for blocking their differentiation into effector cells [86].

In sum, multiple microRNAs control IFN-γ and type 1 differentiation pathway (Table 1.1), which thus constitute important mechanisms to modulate (auto)immune responses therapeutically.

MicroRNA-mediated regulation of IL-17 and type17-associated factors

IL-17 is another important mediator of the pro-inflammatory activity of T cells, being the major driver of the elimination of extracellular bacteria and fungi. Uncontrolled IL-17 responses, however, have been shown to cause strong inflammatory diseases, including colitis and multiple sclerosis [127–131].

IL-17A is the signature cytokine of Th17 cells, which, in addition, can secrete IL-17F and IL-22 after polarization with IL-6, IL-21, IL-23 and TGF-β. The Th17 master transcription factor, RORγt (encoded by *Rorc*) is induced by STAT-3 [59]. Several microRNAs have been identified which promote the differentiation into Th17 cells.

In the murine model of multiple sclerosis (MS), Experimental Autoimmune Encephalomyelitis (EAE), miR-301a manipulation was shown to affect disease severity. MiR-301a was found to promote Th17 cell differentiation by directly targeting PIAS3, a strong inhibitor of the STAT3 pathway thus leading to an increase in IL-17 production. In line with this model, the levels of miR-301a on adoptively transferred myelin-specific CD4$^+$ T cells determined IL-17 abundance in central nervous system (CNS) infiltrates and

the EAE score, making this microRNA a candidate to be used in future therapeutic approaches [104].

MiR-132/212 cluster is also upregulated under Th17-polarising conditions. This upregulation is dependent on aryl hydrocarbon receptor (AHR) and, in turn, the ability of AHR to promote Th17 cell differentiation depends on the expression of miR-132/212 [101]. The fact that miR-132/212 double knock-out murine naïve $CD4^+$ T cells had elevated levels of Bcl-6, a negative regulator of Th17 cell differentiation, together with the ability of miR-212, but not miR-132, to bind to the 3' UTR of Bcl-6, could explain the observed phenotypes [101].

MiR-326 is another microRNA that promotes Th17 differentiation. MiR-326 targets a known negative regulator of Th17 cells, Ets-1, and is implicated in the ontogeny of multiple sclerosis in humans [105]. However, these results are controversial, as subsequent studies could not detect miR-326 in neither murine nor human Th17 cells, and no differential expression was identified in MS patients versus healthy controls [86,132,133].

MiR-155 has described to be part of a complex network regulating Th17 cytokines. MiR-155 is highly expressed in the Th17 cell subset, both in humans and in mice, and its deficiency leads to lower expression of IL-17A and IL-22, as well as IL-9 and IL-10 [134,135]. In a murine model of *Toxoplasma gondii* infection, miR-155 was shown to be required for cytokine expression by Th17 cells and for Treg cell homeostasis, but not for the Th1 response against the parasite [134]. This contrasted with previous reports of miR-155 involvement in *Helicobacter pylori* infection and in EAE, where miR-155 had been suggested to be required for the development of both Th17 and Th1 cell subsets [136,137]. Further studies are required to elucidate the mechanisms behind this differential impact of miR-155 under different infection and inflammation models.

While Th17-driving transcription factors, such as RORγt, BATF, IRF4, were shown not to be targets of miR-155, Jarid2 (Jumonji, A T Rich Interactive Domain 2), a previously described miR-155 target, was shown to accumulate in T cells in the absence of miR-155 [134,138]. Jarid2 is a chromatin binding protein that silences transcription by recruiting PRC2 (Polycomb Repressive Complex 2), which in turn mediates the trimethylation of histone 3 at lysine 27 (H3K27me3). In the absence of miR-155, the increased widespread recruitment of Jarid2 was shown to match the increase in H3K27me3 marks. This increase in H3K27me3 marks in turn correlates with the repression of multiple gene *loci*: *Il22*, *Il10*,

Il9, *Atf3*, *Tbx21* and *Eomes*, but not *Il17a*, *Rorc*, *Batf* or *Irf4* [134]. The authors further showed that Atf3 was capable of promoting IL-17A production in Th17 cells. In line with all these results, Jarid2$^{-/-}$ CD4^{+} T cells showed increased expression of IL-22, IL-10, IL-9, Atf3 and IL-17A but not IFN-γ, RORγt, Irf4 and Batf [134]. This suggests that miR-155 is not involved in the initial stages of Th17 cell development, but rather in their effector functions. Another mechanism by which miR-155 promotes IL-17 production in T cells is by targeting Ets-1, an inhibitory transcription factor in Th17 differentiation [135,139].

In contrast with the previous microRNAs, miR-20b, a member of the miR-106a-363 cluster, has been implicated in the suppression of Th17 differentiation through the direct targeting of the 3' UTRs of RORγt and STAT3 mRNAs [93]. Overexpression of miR-20b inhibited Th17 cell differentiation and reduced EAE severity, which could be restored by co-infecting the mice with a lentivirus expressing the 3' UTRs of RORγt or STAT3 mRNAs [93]. Thus this study adds miR-20b to the growing list of microRNAs with therapeutic potential.

MiR-210 is greatly induced in CD4^{+} T cells upon TCR stimulation, especially under Th17-polarising conditions [103]. Interestingly, miR-210 expression was shown to be controlled by the hypoxia-induced transcription factor HIF-1α, which in turn is a direct target of miR-210, thus establishing a negative feedback loop [103]. HIF-1α enhances Th17-cell differentiation by promoting RORγt transcription and FoxP3 degradation [140]. Deletion of miR-210 promoted Th17 cell differentiation under conditions of limited oxygen, and impacted on the severity of experimental colitis [103].

Another microRNA-dependent mechanism that can limit IL-17 production consists on the inhibition of IL-23R expression, whose 3' UTR is targeted by the microRNA let-7f [108]. Signalling through the IL-23R results in activation of the JAK-STAT pathway and consequently promotes IL-17 production [141]. IL-23R expression was also shown to be reduced in miR-155$^{-/-}$ cells, which were hyperesponsive to IL-23 [135].

From all the studies regarding microRNA-mediated regulation of IL-17 production it is curious to note that none identified IL-17 as direct target of a given microRNA.

In sum, these examples reveal the critical importance of microRNAs in cytokine regulation across multiple functional T cell subsets. We can also conclude that some microRNAs,

including miR-155 and miR-146a, act on different T cell subsets. This suggests that the expression of these pleiotropic microRNAs must be under tight control.

AIMS OF THIS THESIS

2. Aims of this thesis

In this thesis we set out to investigate how microRNAs might impact T cell differentiation outside the mostly studied $CD4^+$ T cell compartment. We thus focused on $CD8^+$ and $\gamma\delta$ T cells and their potent effector cytokine responses.

Aim 1: Identify microRNA regulators of effector $CD8^+$ T cell differentiation.
$CD8^+$ $\alpha\beta$ T cells are essential for host defense against viruses or intracellular bacteria, while also constituting the major effector lymphocyte subset in cancer surveillance. The main effector cytokine produced by $CD8^+$ T cells is IFN-γ, which orchestrates anti-viral and anti-tumoural immune responses. We aimed to identify key microRNAs regulating the IFN-γ program in $CD8^+$ T cells, including their most relevant mRNA targets in effector $CD8^+$ T cell differentiation.

Aim 2: Dissect microRNA-dependent regulation of $\gamma\delta$ T cell effector functions.
Although $\gamma\delta$ T cells are relevant participants in a very broad spectrum of processes, they can be segregated in terms of function into two main groups accordingly to their ability to produce the cytokines IL-17 and IFN-γ. These cytokines are critical in a large number of processes such as infection, tissue homeostasis, autoimmunity and cancer. $\gamma\delta$ T cells assume an important role in many of these processes due to their ability to rapidly secrete considerable amounts of these cytokines. Here we aimed at characterizing what defines the identity of these two functionally distinct $\gamma\delta$ T cell subsets at both the messenger RNA and microRNA levels; and integrate the most relevant candidates in microRNA:mRNA networks of regulation of $\gamma\delta$ T cell differentiation.

Overall, we aimed to provide novel insights into microRNA-mediated regulation of cytokine production by effector T cell subsets, and thus improve our knowledge and capacity to manipulate them in future immunotherapeutic approaches.

MATERIALS AND METHODS

3. Materials and methods

Mice

All mice used were adults 6 to 12 weeks of age. C57BL/6J mice were purchased from The Jackson Laboratory. LckCre Dicer$^{\Delta/\Delta}$ and Dicer$^{lox/lox}$ mice were kindly provided by Matthias Merkenschlager, Imperial College, London. IFN-γ-IRES-YFP-BGHpolyA (YETI) mice were purchased from Biocytogen. C57BL/6-*Il17a*^{tm1Bcgen}/J (IL17A-IRES-GFP-KI) mice were purchased from Biocytogen. B6.129S4-*Ifng*$^{tm3.1Lky}$/J (Great) mice were purchase from The Jackson Laboratory.

Mice were bred and maintained in the specific pathogen–free animal facilities of Instituto de Medicina Molecular (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 Instituto de Medicina Molecular.

Monoclonal antibodies

The following anti-mouse monoclonal antibodies (mAbs) were used (antigens and clones). Fluorescently labelled: CD3ε (145.2C11), CD4 (GK1.5), CD8 (53–6.7), TCRβ (H57-597) TCRδ (GL3), CD44 (IM7), CD45RB (C363.16A), IFN-γ (XMG1.2) and IL-17A (TC11.18H10.1). Purified: CD3 (145-2c11) and CD28 (37.51).

Antibodies were purchased from BD Biosciences, eBiosciences or BioLegend.

Cell preparation, flow cytometry and cell sorting

Cell suspensions were obtained from spleens, lymph nodes or thymus. Erythrocytes were osmotically lysed in red blood cell lysis buffer (BioLegend). Cells were filtered through 70 μm cell strainers (BD Biosciences).

For cell surface staining, single-cell suspensions were incubated for 30 min with saturating concentrations of mAbs (see above).

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 (10 μg/ml) (all from Sigma-Aldrich) for 3-4 h at 37 °C. Cells were stained for the above identified cell surface markers, fixed 30 min at 4 °C, permeabilized with the Foxp3 Transcription Factor Staining Buffer set (eBioscience) in the presence of anti-

CD16/CD32 (eBioscience) for 15 min at 4 °C, and lastly incubated for 30 min-1 h at 4 °C with the above identified antibodies in permeabilization buffer.
Samples were analysed using LSRFortessa (BD Biosciences) and FlowJo software (Tree Star). For sorting, cells were prepared and stained for cell surface markers as mentioned above and sorted on a FACSAria (BD Biosciences). If cells were to be used for RNA extraction they were washed with cold PBS before being frozen in liquid nitrogen.

microRNA qPCR profiling

All experiments were conducted at Exiqon Services. The submitted RNA was reverse transcribed in 40 µl reactions using the miRCURY LNA Universal RT miRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). cDNA was diluted 50 times and assayed in 10 µl PCR reactions according to the protocol for miRCURY LNA Universal RT miRNA PCR; each microRNA was assayed once by qPCR on the miRNA Ready-to-Use PCR, Rodent panel I. Negative controls excluding template from the reverse transcription reaction was performed and profiled like the samples. The amplification was performed in a LightCycler 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analysed using the Roche LC software, both for determination of Cp (by the 2nd derivative method) and for melting curve analysis. The amplification efficiency was calculated using algorithms similar to the LinReg software. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for the assay. Furthermore assays must be detected with 5 Cp's less than the negative control, and with Cp<37 to be included in the data analysis. Data that did not pass these criteria were omitted from any further analysis. Using NormFinder the best normalizer was found to be the average of assays detected in all samples. All data was normalized to the average of assays detected in all samples (average – assay Cp).

RNA isolation, complementary DNA production, and qPCR

Total RNA (including mRNA and small RNA) was isolated using miRNeasy Mini kit (Qiagen). Messenger RNA was subsequently reverse transcribed with random oligonucleotides (Invitrogen) using Moloney murine leukemia virus reverse transcriptase

(Promega). Extracted microRNA was subject to reverse transcription with miRCURY LNA Universal RT miRNA system (Exiqon). Both type of molecules were amplified by quantitative PCR using SYBR green (Applied Biosystems) on a ViiA 7 Real-Time PCR system (Applied Biosystems; Life Technologies). Primers for mRNA were designed with Primer Blast or via the Universal ProbeLibrary Assay Design Center (Roche) being their sequences available upon request. miRNA LNA PCR primer sets were purchased from Exiqon. Analysis of quantitative PCR results was performed using the ViiA 7 software v1.2 (Applied Biosystems; Life Technologies). Results were normalized to the following references: mir-423-3p or RNA RNU5G for microRNA quantification; *efa1* or *actb* and *b2m* for mRNA quantification.

CD8$^+$ T cell electroporation

CD3$^+$ CD8$^+$ T cells were sorted from lymph nodes and spleens of C57BL/6J mice and stimulated with plate-bound anti-CD3 and anti-CD28 (both at 2.5 µg/ml) for 48 h. Cells were then resuspended in T buffer and used (200000 cells per condition) for transfection of mimics with the Neon electroporation transfection system (Invitrogen) using the 10 µl tip and applying the following parameters: 3 pulses of 10 ms and 1550 V. After electroporation, cells were cultured in media supplemented with IL-2 (1 ng/ml) and 48 hours later stimulated for intracellular cytokine staining. The mimics were used at 500 nM per electroporation. Mimics of mir-132-3p, mir-139-5p, mir-181a-5p, mir-200a-3p, mir-322-5p, mir-451 and negative control are all miRCURY LNA miRNA mimics from Exiqon. The siRNA against Id2 was obtained from Dharmacon and use at a concentration of 20-500 nM.

$\gamma\delta^+$ T cell electroporation

CD3$^+$ $\gamma\delta^+$ T cells were sorted from lymph nodes and spleens of C57BL/6J mice and stimulated with plate bound anti-TCR$\gamma\delta$ and cultured accordingly to the protocol in **Figure 4.2.13**. Prior to electroporation cells were resuspended in T buffer and used (200000 cells per condition, 25000 per electroporation) for transfection of mimics/inhibitors with the Neon electroporation transfection system (Invitrogen) using the 10 µl tip and applying the following parameters: 3 pulses of 10 ms and 1550 V. After electroporation, cells were cultured for 48 h prior to stimulation for intracellular cytokine

staining. The mimics and inhibitors were both used at 1 μM per electroporation. Mimics and inhibitors were purchased from Dharmacon. Cytokines and antibodies were used at the following concentrations: anti-TCR$\gamma\delta$ (1 μg/ml),anti-IFN-γ (10 μg/ml), IL-23 (5 ng/ml), IL-1β (5 ng/ml) and IL-7 (20 ng/ml).

$\gamma\delta^+$ T cell transduction

$CD3^+$ $\gamma\delta^+$ T cells were sorted from lymph nodes and spleens of C57BL/6J mice and stimulated with plate bound anti-TCR$\gamma\delta$ and cultured accordingly to the protocol in **Figure 4.2.14** . Cells were transduced by a 90 min centrifugation at 30 ˚C in the presence of viral supernatant and polybrene (8 μg/ml, Sigma.Aldrich). Cytokines and antibodies were used at the following concentrations: anti-TCR$\gamma\delta$ (1 μg/ml),anti-IFN-γ (10 μg/ml), IL-23 (5 ng/ml), IL-1β (5 ng/ml) and IL-7 (20 ng/ml).

Viral particle production

The retroviral constructs encoding candidate microRNAs' were generated by inserting the respective native pre-miRNA sequences flanked by about 200 bp into a modified pMIG retroviral vector (pMig-PGW).

Retroviral particles were produced in HEK 293T TAT cells co-transfected with the plasmid containing the microRNA and a packaging vector. Supernatant containing the viral particles was concentrated and collected by high speed centrifugation and kept at -80 ˚C.

Cell culture

Lymphocytes were cultured in RPMI medium supplemented with 10% FBS, 1% HEPES, 1% non-essential aminoacids (NEAA), 1% Sodium pyruvate (NaPu), 1% penicillin and streptomycin (Pen/Strep), 0.1% Gentamicin and 0.1% β-mercaptoethanol.

HEK 293T TAT were cultured in DMEM (high glucose, pyruvate) supplemented with 10% FBS.

All cells were incubated at 37 ˚C and 5% CO_2. All media and supplements were purchased from Gibco.

MicroRNA's target gene prediction analysis

Predicted targets, validated targets and target binding sites for the microRNAs of interest were determined using the online platforms of miRWalk2.0 [142] or DIANA tools (namely microT-CDS v5.0 and TarBase v.8) [143,144]. For target prediction several algorithms were used simultaneously (namely MirWalk, miRanda, targetScan e RNA22).

Next Generation Sequencing (NGS)

NGS was performed at the EMBL by the Genomics Core Facility staff. Briefly the total RNA sent was tested for quality (RIN) and used for the generation of both the mRNA library and the microRNA library. Sequencing was performed in an Illumina HiSeq2000 platform.

NGS data analysis

Analysis was performed in collaboration with Daniel Sobral from Instituto Gulbenkian de Ciência. Two different pipelines were used for mRNA and microRNA analysis and are outlined in **Figure 2.1**. Summarily, sequences were analysed and filtered according to their quality, after which the sequencing adapters were removed. Sequences were then aligned with the genome to generate gene counts, which were the used determine differential expression with the edgeR package [145,146].

Gene Ontology (GO) term and Pathway enrichment analysis

Enrichment analysis was performed using TargetMine online platform (https://targetmine.mizuguchilab.org/) [147,148] where a one-tailed Fisher's exact test is used for different biological themes or pathways.

Statistical analysis

GraphPad Prism 7.0 was used for statistical analysis. The statistical significance of differences between populations was assessed using either the two-tailed nonparametric Mann-Whitney test or the column statistics one-sample t test when applicable. P-values under 0.05 were considered significant. In bar graphs, data is always presented as mean ± SD.

Statistics of the analysis of the microRNA and mRNA profiles is described elsewhere.

* $p \leq 0.05$ ** $p \leq 0.01$.

Figures

Graphs were created using GraphPad Prism 7.0.

MicroRNA:mRNA networks were created using the NAViGaTOR 2.3.0 software.

Pathways schematics were done using KEGG Mapper - Color Pathway.

Some of the figures were created using the vector image bank of Servier Medical Art (http://smart.servier.com/) (https://creativecommons.org/licenses/by/3.0/).

a **b**

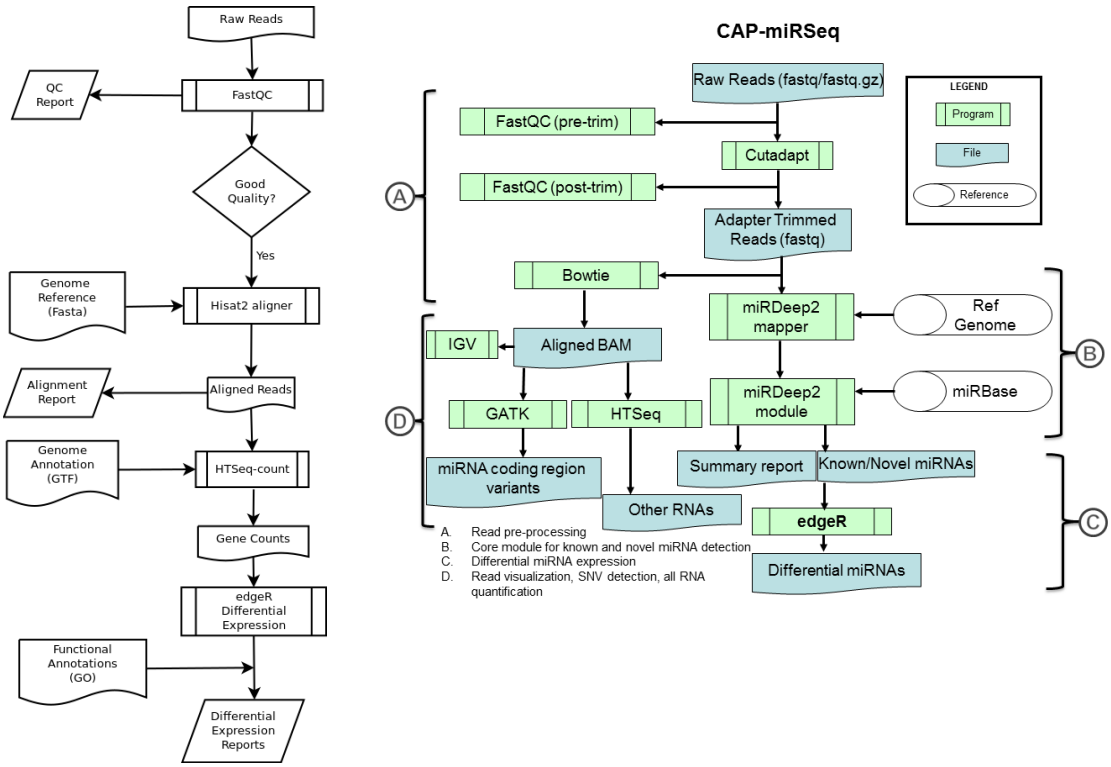


Figure 2.1 - Pipeline used for the analysis of NGS data.

Graphic representation of the steps followed to analyse the data obtained from (**a**) mRNA NGS and (**b**) microRNA NGS that led differential expression reports used for subsequent analysis.

RESULTS

4. Results

4.1 - Post-transcriptional regulation of IFN-γ production by CD8⁺ T cells

This section was submitted for publication in J Mol Med (under review):
MicroRNAs 181a and 451a dampen a signalling hub that regulates IFN-γ production by effector CD8⁺ T cells
Tiago Amado, Ana Amorim, Paula V. Romero, Daniel Inácio, Francisco Enguita, Nina Schmolka*, Bruno Silva-Santos* and Anita Q. Gomes*

4.1.1 - Introduction

4.1.1.1 - IFN-γ production by CD8⁺ T cells

Interferon-γ (IFN-γ) is a critical cytokine in immunity against viral and intracellular bacterial infections as well as for tumour control. Studies with genetically modified mice lacking IFN-γ responses (with either *Ifng* or *Ifng* gene receptor 1 disruptions) have clearly shown a high susceptibility to bacterial, protozoan and viral infections [149]. Moreover, when challenged with chemical carcinogens, IFN-γ-deficient mice develop more tumours, and more rapidly, than wild type animals [113,150].

CD8⁺ T cells are a key source of IFN-γ within the adaptive immune response; and play crucial roles in the control of intracellular infections and tumourigenesis [151–154]. Consistent with this, studies enhancing the production of IFN-γ by CD8⁺ T cells have shown improved anti-tumour responses *in vivo* in several mouse models of cancer; and the robust activation of human CD8⁺ T cells, including an IFN-γ molecular signature, are thought to underlie the recent successes of checkpoint inhibitors in cancer treatment [155–157].

After antigen recognition, activated CD8⁺ T cells undergo proliferative expansion and differentiate into cytotoxic T lymphocytes (CTLs) that are able to produce effector molecules, among which IFN-γ and the cytotoxicity mediators perforin and granzyme B

[151]. IFN-γ is the key orchestrator of the CTL response, since it not only boosts cytotoxicity but also upregulates the expression of MHC class I that is critical for antigen recognition and activation of $CD8^+$ T cells [149].

The induction of IFN-γ expression is a tightly regulated process in effector $CD8^+$ T cell differentiation. At steady state, naïve $CD8^+$ T cells produce little IFN-γ, but there is a marked upregulation upon TCR activation, with synergistic inputs from CD27 and CD28 co-receptors and IL-12 and IL-18 signals [158,159].

Downstream of cell surface signals, the process is controlled at the transcriptional level, where the transcription factors T-bet and Eomesodermin (Eomes) play the central roles [160,161]. These seemingly play complementary roles in $CD8^+$ T cell differentiation, since T-bet expression associates with effector phenotype whereas Eomes levels increase in memory $CD8^+$ T cells [161].

4.1.1.2 - MicroRNA-mediated regulation of effector functions in $CD8^+$ T cells

Concomitant with major transcriptional changes, $CD8^+$ T cell differentiation has been recently associated with microRNA-mediated post-transcriptional regulation. MicroRNAs are required for the correct development, homeostasis and function of $CD8^+$ $\alpha\beta$ T cells [76,77]. Absence of Dicer reduces production and/or survival of CD8 single positive (SP) thymocytes and also leads to a marked reduction in the number of $CD8^+$ T cells found outside the thymus, namely in the spleen, lymph nodes and blood. Curiously their numbers seem to be more affected than those of their $CD4^+$ T cell relatives [77]. Dicer is also necessary for normal effector $CD8^+$ T cell expansion in response to a pathogenic infection *in vivo* [162]. MicroRNAs seemingly restrain the activation and acquisition of effector functions by activated $CD8^+$ T cells, as indicated by the increased production of cytotoxic molecules in mouse $CD8^+$ T cells genetically depleted of the microRNA processing enzyme, Dicer; and in human $CD8^+$ T cells where Dicer was knocked-down by the means of RNA interference [163]. Although various individual microRNAs have been identified either as positive or as negative regulators of $CD8^+$ T cell differentiation *in vivo*, from the

observation of a general trend of global microRNA downregulation in the passage from naïve to effector cells and posterior increase in expression levels upon passage to memory phenotype, some profiling studies have also brought forward this notion that, in general, microRNAs limit $CD8^+$ T cell differentiation or activation [164,165]. Along with this line of thought we find microRNA let-7, whose high levels in naïve cells help maintain that phenotype but then drop upon TCR activation leading to the de-repression of its targets, that include Myc and Eomes, enhancing clonal expansion and the acquisition of effector functions [166]. Down-regulation of let-7 thus promoted anti-viral and anti-tumoural $CD8^+$ T cell responses [166].

In a similar fashion, upon activation, the expression levels of miR-29 are reduced in $CD8^+$ T cells, through NF-κB mediated transcriptional suppression, which facilitates the production of miR-29 target IFN-γ, essential for the host resistance to infection [99]. MiR-139, found down-regulated by inflammation in CTLs, has also been implicated in effector cell differentiation, by its ability to target Eomes and perforin [163].

Another microRNA that limits the action of $CD8^+$ T cells, especially in a tumour microenvironment where TGF-β is present, is miR-491. TGF-β can induce expression of miR-491 and elevated levels of this microRNA limit cellular proliferation, promote apoptosis and decrease IFN-γ expression, thus compromising the ability of these cytotoxic lymphocytes to fight cancer cells. MiR-491 acts as a negative regulator of T cells by inhibiting genes like cyclin-dependent kinase 4 (Cdk4), the anti-apoptotic protein B-cell lymphoma 2-like 1 (Bcl-xL) and the transcription factor T cell factor 1 (Tcf-1) [167]. In parallel, TGF-β can also suppress CTLs' anti-tumour activity by inducing the expression of another microRNA, miR-23a. By targeting Blimp-1, miR-23a is able to restrain $CD8^+$ T cell differentiation and cytotoxicity. In accordance with this, CTLs extracted from a tumour microenvironment that showed deficient cytotoxic activity had elevated levels of this microRNA and highly toxic CTLs show lower levels of miR-23a than poorly toxic CTLs [95,168] . Moreover, blocking miR-23 enhanced granzyme B expression in human $CD8^+$ T cells; and inhibited tumour progression in a mouse model of cancer [168].

Although most microRNAs have higher levels in naïve and memory cells and are only present in effector cells at very low levels, there are some, like miR-17-92 or miR-155 amongst others, that behave in an opposite way, acting as promoters of $CD8^+$ T cell differentiation.

Mir-21 is frequently found elevated in T cells under inflammatory contexts and its expression is highest in effector T cells and lowest in naïve T cells suggesting that miR-21 may play an important role in maintaining the effector stage of the T cells [70,165,169]. Consistently, its overexpression in T cells resulted in an increase production of the pro-inflammatory cytokines IFN-γ and TNF-α and, it is proposed that higher levels miR-21 may enable T cells to elude activated T cell apoptosis and enhance pro-inflammatory cytokine secretion by its ability to target PDCD4 expression [170].

Besides cytotoxicity, microRNAs can also impact effector $CD8^+$ T cell proliferation and memory cell differentiation, as shown for the miR-17-92 cluster in the context of viral infection [171]. Expression levels of miR-17-92 undergo dynamic changes in the course of a $CD8^+$ T cell response. In an acute viral infection, miR-17-92 expression is strongly induced after T cell activation, downregulated upon the end of clonal expansion and further reduced, to levels seen in naïve cells, during the memory development phase. MiR-17-92 promotes effector differentiation, metabolism and the cell-cycle progression of effector $CD8^+$ T cells, essential for the optimal and fast expansion of these cells upon challenge. This is likely accomplished by targeting Pten, Pdcd1 and other several negative regulators of the PI3K-AKT-mTOR pathway. Failure to reduce miR-17-92 levels when the acute phase is passed leads to a defective development of memory cells making this microRNA cluster important for both the control of effector cells expansion but also for memory cell formation during a viral infection [70,171,172].

Still in the line with the same logic, $CD8^+$ T cells deficient in miR-155 have a decreased potential to differentiate into effector cells in response to viral infection, reflected in a significant increase in the organism's viral load. In addition to viral replication, miR-155-deficient $CD8^+$ T cells were also ineffective at controlling tumour growth, while cells with miR-155 overexpression enhanced the anti-tumoural response *in vivo*. MiR-155 enhances $CD8^+$ T cells effector functions by repressing Ship1, a strong inhibitor of the transcription factor T-bet and it's transcriptional target IFN-γ, Akt and negative regulators of Stat5 such as Socs1 and Ptpn2. MiR-$155^{-/-}$ $CD8^+$ T cells also show enhanced type I interferon signalling [119,173–177].

$CD8^+$ T cells without miR-150 also fail to undergo robust expansion and differentiation into effector cells in response to infection as this microRNA targets several genes associated with proliferation or effector functions [178]. MiR-150 has also been implicated in

memory formation, reducing it if overexpressed, likely occurring as a consequence of the reduction of one of it's targets levels, c-Myb [179].

Albeit also being elevated in effector cells, miR-31 does not stimulate effector functions, by the contrary, its presence promotes $CD8^+$ T cell dysfunction and hinders their performance. MiR-31 levels are strongly induced following TCR activation in a pathway dependent on calcium and on activation of the transcription factor NFAT. MiR-31 increases the sensitivity to type I interferons and promotes T cell dysfunction by increasing the abundance of several inhibitory molecules such as c-Maf, prostaglandin E2 receptor (Ptger2) and metallothioneins, also promoting $CD8^+$ T cell exhaustion during the chronic stage of viral infection [180] .

MiR-146a is another example of a microRNA acting as a break to immune responses. $CD8^+$ T cells lacking miR-146a display hyperresponsiveness upon TCR stimulation: exaggerated activation, higher proliferation, lengthened survival and augmented effector cytokine production. Conversely, cells with excess miR-146a show the opposite phenotype. MiR-146a expression is highly upregulated upon cell activation in an NF-κB-dependent manner. In turn, miR-146a is able to downregulate NF-κB activity by repressing the signal transducers Traf6 and Irak1, thus establishing a negative feedback loop [123,124,181,182]. Disruption of this feedback loop leads to autoimmunity and premature death, as a result of the hyperactive miR-146a$^{-/-}$ $CD8^+$ T cells that display abnormal effector cytokine secretion, increased proliferation and reduced apoptosis [123].

While some of these previous studies established the importance of various microRNAs in $CD8^+$ T cell (cytotoxic) responses *in vivo*, here we aimed at a focused dissection of microRNAs that may specifically regulate IFN-γ expression in $CD8^+$ T cells.

4.1.2 - Results

4.1.2.1 - MicroRNA-deficient CD8⁺ T cells overexpress IFN-γ

This study initiated with the observation of surprisingly high IFN-γ protein expression in thymic CD8⁺ T cells from a conditional Dicer-deficient mouse strain, controlled by the proximal *Lck* promoter, which therefore deleted Dicer at early stages of thymic T cell development [76]. Thus, CD8⁺ thymocytes from Dicer-deficient (lckCre dicer$^{\Delta/\Delta}$) mice contained almost 40% of IFN-γ-producing cells (upon short-term PMA plus ionomycin restimulation), compared to ~3% of IFN-γ⁺ CD8⁺ thymocytes in Dicer-proficient mice (dicer$^{lox/lox}$) (**Figure 4.1.1 a-b**). Consistent with this finding at the protein level, the mRNA expression levels of *Ifng* and its two main transcriptional regulators, *Tbx21* and *Eomes*, were up-regulated in Dicer-deficient CD8⁺ T cells when compared to controls (**Figure 4.1.1 c**). These data revealed a striking impact of the Dicer-dependent microRNA machinery on IFN-γ expression in thymic CD8⁺ T cells; and beckoned the identification of specific microRNAs underlying this phenomenon.

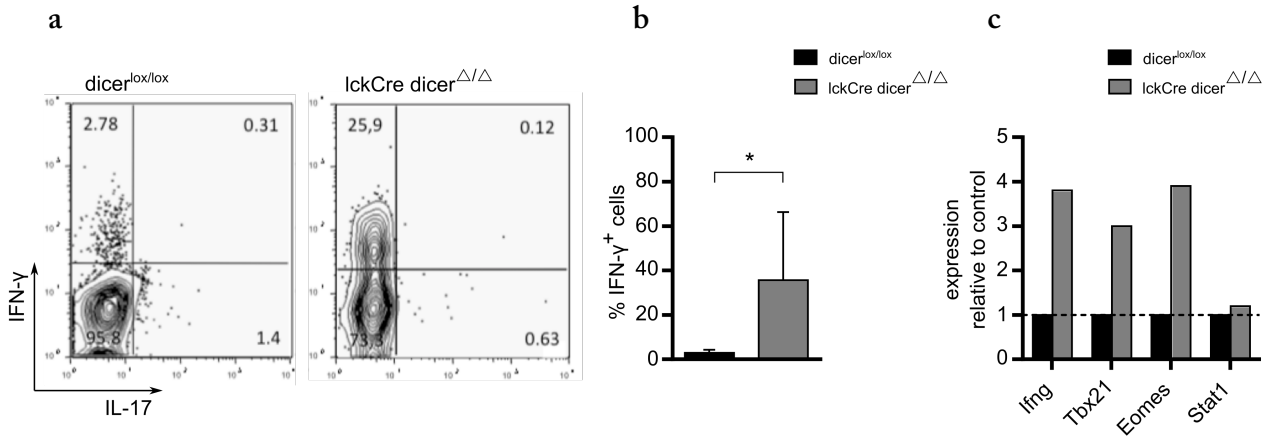


Figure 4.1.1 - Dicer-deficient thymic CD8⁺ T cells overexpress IFN-γ.
Representative plot (**a**) and quantification (**b**) of flow cytometry analysis of IFN-γ and IL-17 protein levels in thymic CD8⁺ T cells of dicer$^{lox/lox}$ and lckCre dicer$^{\Delta/\Delta}$ (n=4). (**c**) RT-qPCR analysis of *Ifng*, *Tbx21* and *Stat1* in thymic CD8⁺ T cells of dicer$^{lox/lox}$ relative to lckCre dicer$^{\Delta/\Delta}$ mice.

4.1.2.2 - *Differential microRNA expression analysis identifies candidates segregating with IFN-γ expression in CD8⁺ T cells*

To identify individual microRNAs that might regulate IFN-γ expression in CD8⁺ T cells, we compared the miRNome of cells either positive or negative for *Ifng locus* activity in the reporter mouse strain, IFN-γ-IRES-YFP-BGHpolyA knock-in, also known as YETI (from Biocytogen) [23].

Upon FACS isolation of YFP⁺ (IFN-γ⁺) and YFP⁻ (IFN-γ⁻) CD8⁺ T cells from the thymus of YETI mice (**Figure 4.1.2 a**), we extracted RNA, converted it to cDNA and subjected it to qPCR profiling with miRCURY LNA™ Universal RT microRNA PCR Rodent panel I (Exiqon). From the 121 microRNAs identified in all samples, 29 were differentially expressed (at least 2-fold different) between YFP⁺ and YFP⁻ CD8⁺ T cells, as depicted in the heatmap of **Figure 4.1.2 b**. Upon qPCR profiling validation with an independent sample (**Figure 4.1.2 c**), we selected the top up-regulated microRNAs in YFP⁻ CD8⁺ T cells (miR-322, miR-181a and miR-132) and the top up-regulated microRNAs in YFP⁺ CD8⁺ T cells (miR-139, miR-451 and miR-200) for subsequent functional studies.

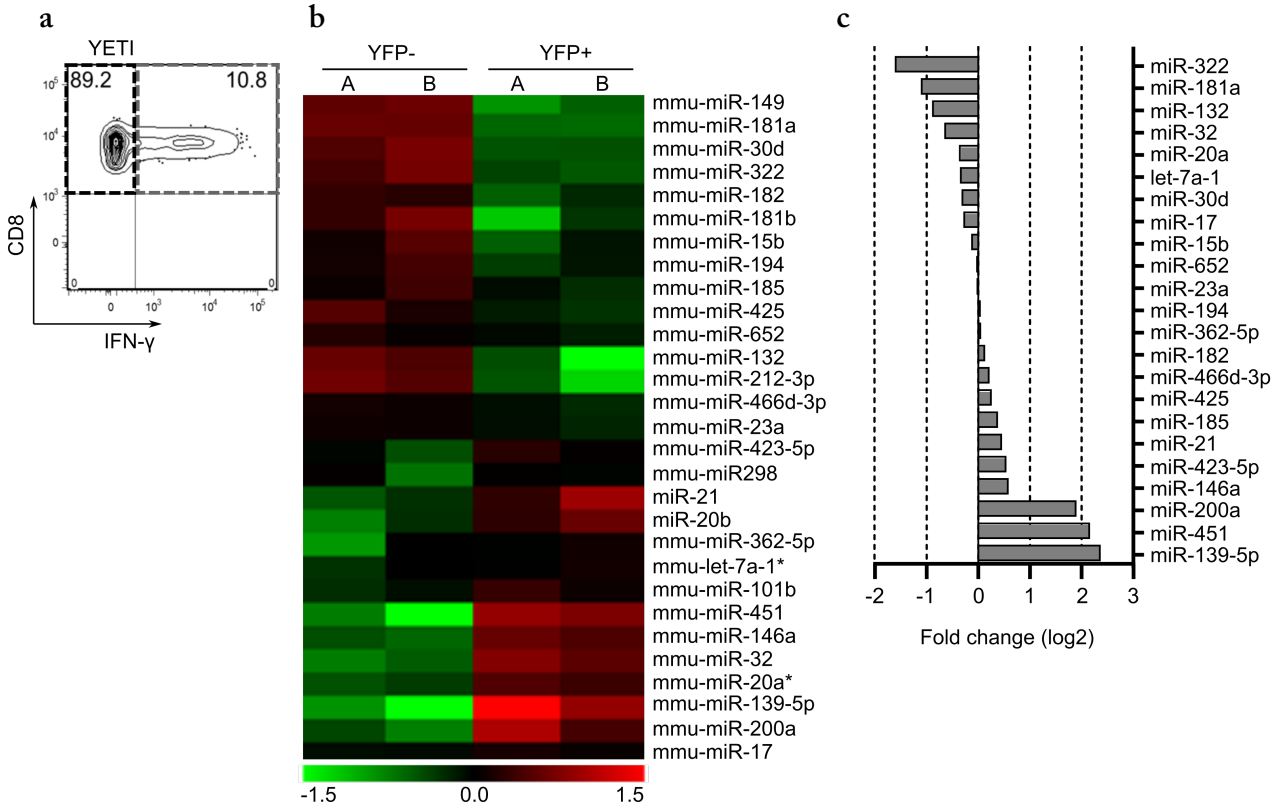


Figure 4.1.2 (shown on preceding page) - Differential expression analysis of microRNAs segregating with IFN-γ expression in CD8⁺ T cells.
(**a**) Isolation by FACS of YFP⁻ *vs* YFP⁺ thymic CD8⁺ T cells from YETI mice for qPCR profiling. (**b**) Heatmap of differentially expressed genes between YFP⁻ and YFP⁺ CD8⁺ thymic T cells. The colour scale shown at the bottom illustrates the relative expression level of a microRNA across all samples: red colour represents an expression level above mean, green colour represents expression lower than the mean. (**c**) qPCR validation of selected microRNA genes' differential expression between IFN-γ producing *vs* non-producing thymic CD8⁺ T cells. MicroRNAs enriched in YFP⁻ have negative log2 fold change values and microRNAs enriched in YFP⁺ have log2 fold change positive values.

4.1.2.3 - *MiR-181a-5p and miR-451a limit IFN-γ production by CD8⁺ T cells*

To perform functional assays with candidate microRNAs we turned to peripheral CD8⁺ T cells. First, we assessed whether the IFN-γ phenotype observed in the Dicer-deficient case: CD8⁺ T cells isolated from pooled spleen and lymph nodes from lckCre dicer$^{\Delta/\Delta}$ mice showed markedly enhanced IFN-γ production (**Figures 4.1.3 a-b**) and *Tbx21*, *Eomes* and *Stat1* expression (**Figure 4.1.3 c**) when compared to control (dicer$^{lox/lox}$) CD8⁺ T cells.

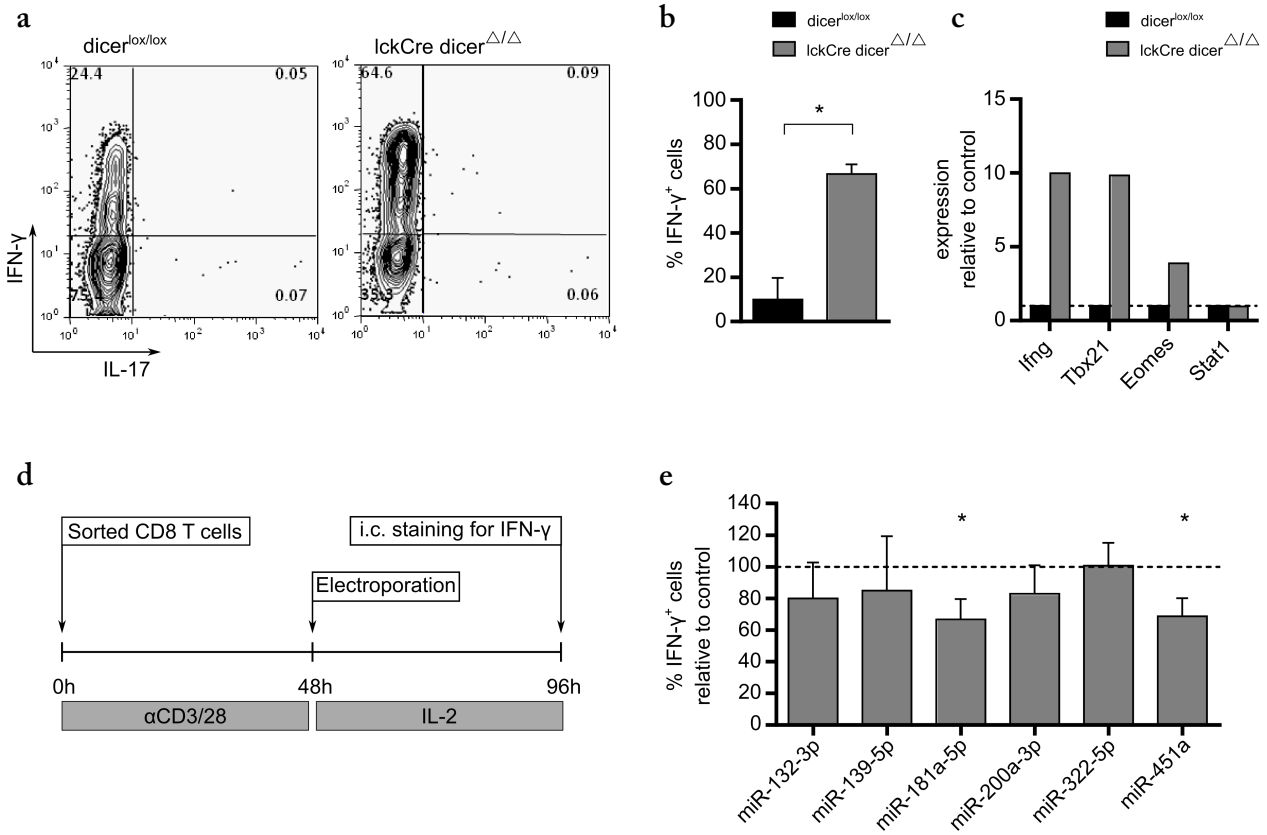


Figure 4.1.3 (shown on preceding page) - MiR-181a-5p and miR-451a limit IFN-γ production by peripheral CD8⁺ T cells.
Representative plot (**a**) and quantification (**b**) of flow cytometry analysis of IFN-γ and IL-17 protein levels in peripheral CD8⁺ T cells of dicer$^{lox/lox}$ and lckCre dicer$^{\Delta/\Delta}$ (n=4). (**c**) RT-qPCR analysis of *Ifng*, *Tbx21*, *Eomes* and *Stat1* in peripheral CD8⁺ T cells of dicer$^{lox/lox}$ relative to lckCre dicer$^{\Delta/\Delta}$ mice. (**d**) Representation of the workflow followed for electroporation of microRNA mimics and (**e**) its effects on reducing IFN-γ protein levels (n=5 to 7).

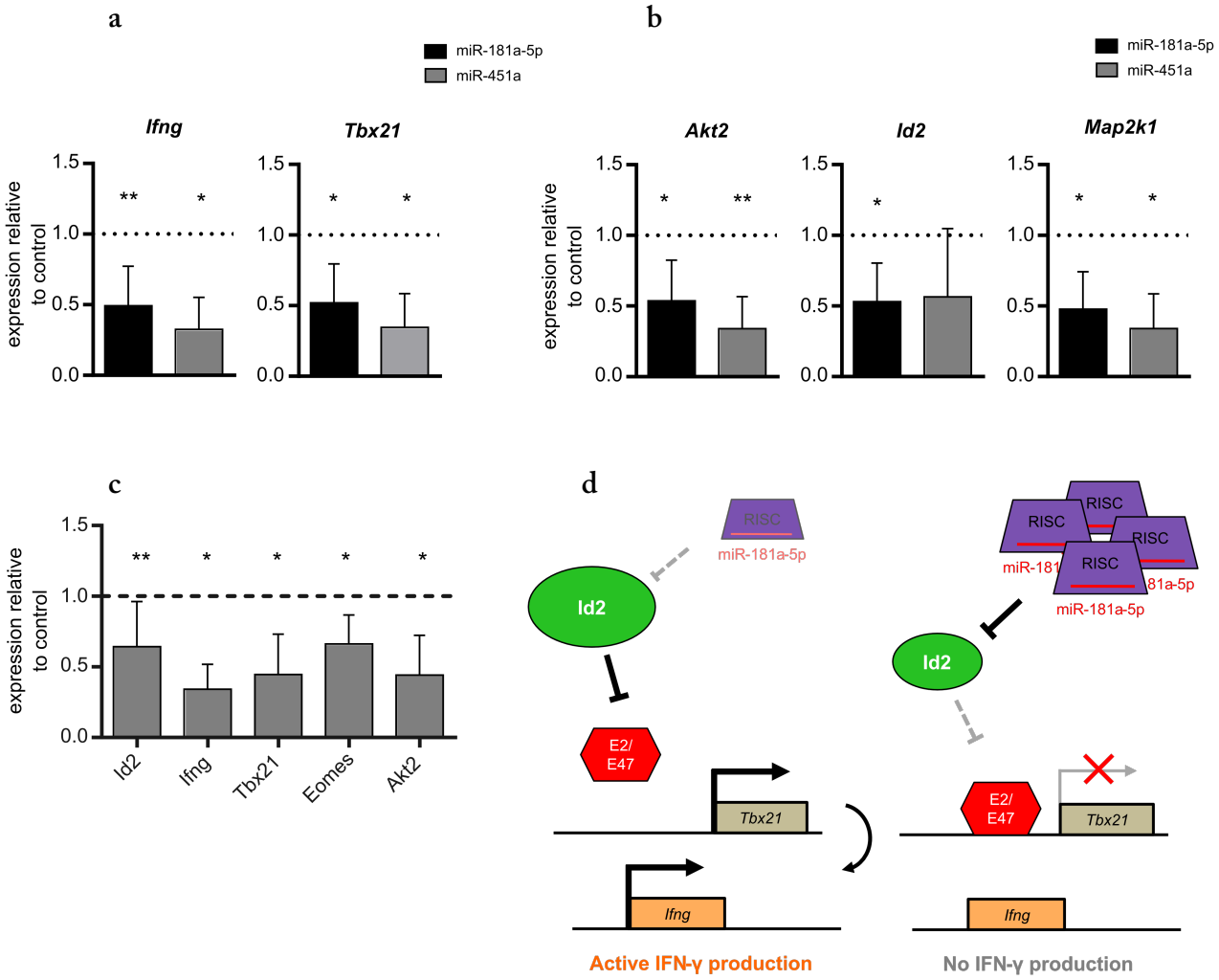
We next performed gain-of-function studies by electroporating synthetic microRNA mimics in CD8⁺ T cells isolated from pooled spleen and lymph nodes. These were cultured for two days with plate-bound anti-CD28 and anti-CD3 monoclonal antibodies and subsequently electroporated with either control or specific mimics (Exiqon) for the six selected microRNAs (from **Figure 4.1.2 c**). After the electroporation, cells were cultured for two extra days in the presence of IL-2, and IFN-γ production was determined by intracellular staining (**Figure 4.1.3 d**). We found that miR-181a-5p and miR-451a, unlike the other microRNA candidates, significantly decreased IFN-γ production when compared to control mimics (**Figure 4.2.3 e**). These results identify miR-181a-5p and miR-451 as new microRNA regulators of IFN-γ production by CD8⁺ T cells.

4.1.2.4. - *MiR-181a-5p dampens an IFN-γ-promoting signalling hub in CD8⁺ T cells*

To understand how miR-181a-5p and miR-451 could impact CD8⁺ T cell differentiation and IFN-γ production, we next aimed at identifying their relevant mRNA targets in this process. We used bioinformatics to assess predicted and validated target mRNAs, thus containing binding sites for miR-181a-5p or miR-451a in their 3' UTR. From these, we selected a set of genes, namely signalling molecules or transcription factors, based on their relevance for the IFN-γ expression program according to the literature (**Figure 4.1.4 a-b** and **Figure 4.1.5 a-b**). To test whether their expression levels were modulated by miR-181a-5p or miR-451a, we electroporated the corresponding mimics in CD8⁺ T cells and subsequently performed RT-qPCR for the putative mRNA targets. Consistent with the IFN-γ protein data (**Figure 4.1.3 e**), the mRNA expression levels of both *Ifng* and its master transcription regulator *Tbx21* were reduced upon miR-181a-5p and miR-451a

mimics transfection (**Figure 4.1.4 a**). Importantly, of the 11 potential direct mRNA targets of these microRNAs that we investigated, only 3 were significantly down-regulated: *Id2*, *Akt2* and *Map2k1*, all of which are only targets for miR-181a-5p (**Figure 4.1.4 b** and **4.1.5 a-b**). These are important signalling components of the IFN-γ expression program: Akt2 and Map2k1 are signal transducing kinases, and Id2 is a key transcription factor in this process [183]. Of note, these results defined specific signalling mediators of the IFN-γ program as being regulated by miR-181a-5p, since for example *Akt2* but not *Akt3*, and *Map2k1* but not *Mapk1* (encoding Erk) nor *Map3k7*, were impacted in the gain-of-function experiments (**Figure 4.1.4 b** and **Figure 4.1.5 a-b**).

Figure 4.1.4 (shown on preceding page) - MiR-181a-5p dampens an IFN-γ-promoting signalling hub in CD8⁺ T cells.

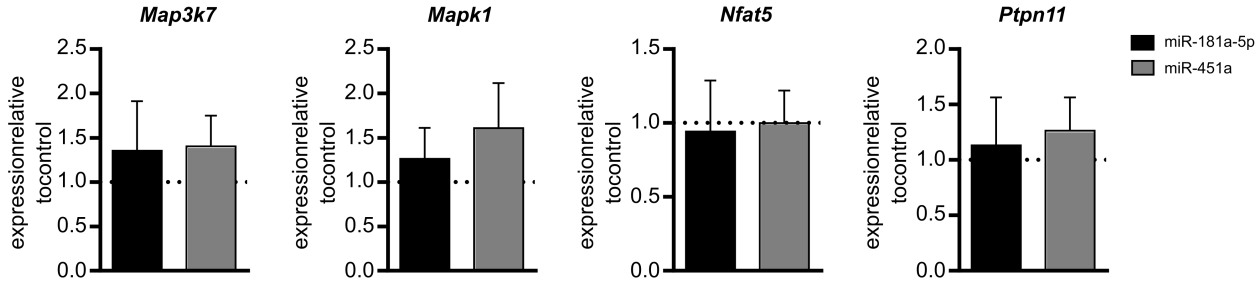


RT-qPCR analysis of (**a**) signature genes *Ifng* and *Tbx21* and (**b**) microRNAs' target genes *Akt2*, *Id2* and *Map2k1* upon transfection of cells with either miR-181a-5p or miR-451a mimics (n=4). (**c**) RT-qPCR analysis of *Id2*, *Ifng*, *Tbx21*, *Eomes* and *Akt2* genes after siRNA-mediated Id2

knockdown (n=4). (**d**) Proposed working model for the interaction between miR-181a-5p and Id2 in the control of IFN-γ expression.

Finally, to perform a functional microRNA: mRNA validation experiment, we selected Id2 since it is a molecularly validated target of miR-181a-5p [184,185] that has been well established as an important regulator of CD8$^+$ T cell differentiation as well as IFN-γ expression [183–187]. We therefore used RNA interference (siRNA) to down-regulate (~40% relative to control levels) Id2 in peripheral CD8$^+$ T cells, and observed a similar phenotype to the miR-181a-5p gain-of-function (**Figure 4.1.4 a-b**), i.e., the reduction in *Ifng*, *Tbx21* and *Akt2* levels (**Figure 4.1.4 c**). These data demonstrate that the miR-181a-5p : Id2 pathway regulates IFN-γ expression in CD8$^+$ T cells; and provide a functional validation to our approach to identify novel microRNA determinants of this biological process.

a



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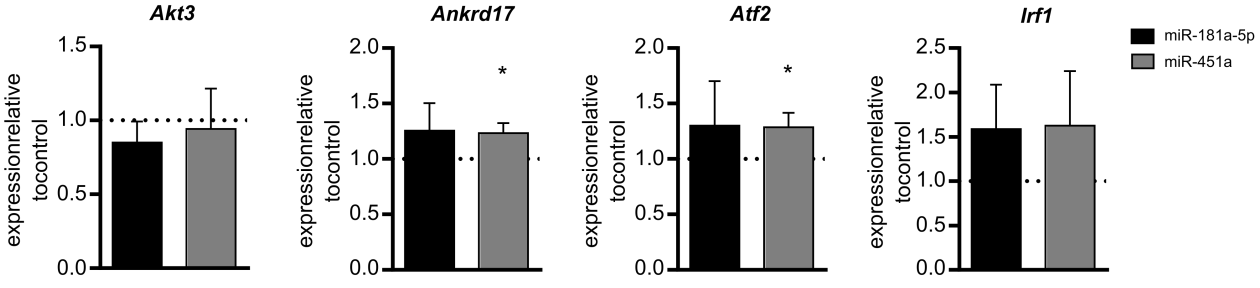


Figure 4.1.5 (shown on preceding page) - Putative mRNA targets not impacted by miR-181a-5p or miR-451a mimics transfection into CD8$^+$ T cells.
RT-qPCR analysis of miR-181a-5p (**a**) or miR-451a (**b**) putative target genes upon transfection of cells with either miR-181a-5p or miR-451a mimics (n=4). Akt3 is predicted to be a target of both these microRNAs.

Collectively, our results establish miR-181a-5p and miR-451a as negative regulators of a IFN-γ expression in CD8$^+$ T cells. Our data also revealed that miR-181a-5p exerts its action by inhibiting a signalling "hub", composed by Akt2, Map2k1 and especially Id2, that promotes IFN-γ expression in CD8$^+$ T cells.

4.1.3 - Discussion

Our study revealed that microRNAs constitute a key developmental brake to IFN-γ expression in CD8$^+$ T cells, since a large fraction of Dicer-deficient CD8$^+$ thymocytes (~40%) were able to produce the cytokine upon short-term restimulation, in stark contrast to control Dicer sufficient cells (~3%). This accumulation of IFN-γ-producing CD8 T cells in the thymus is reminiscent of previous reports on signalling mutant strains, including *Itk*, *Klf2*, *Cbp* and *Id3* deficient mice, where CD8$^+$ T cells with memory-like phenotype and rapid IFN-γ responsiveness, in the absence of antigen exposure, have been termed "innate-like" and shown to participate in the early response against viral and intracellular bacteria infections [159,188–191]. We therefore propose that microRNAs are a major brake to the development of such thymic-derived "innate-like" CD8$^+$ T cells, thereby promoting the adaptive mode of CD8$^+$ T cell response upon activation (and differentiation) in the periphery.

The impact of microRNAs on CD8$^+$ T cells clearly extends beyond IFN-γ expression. On one hand, the deletion of Dicer using the distal *Lck* promoter, which drives Cre expression after the stage of positive selection, resulted in robust responses to activation *in vitro*, but the incapacity to sustain survival and accumulation *in vivo* upon acute infection [162]. On the other hand, the deletion of Dicer in activated CD8$^+$ T cells caused a significant up-regulation of the killing mediators, perforin and granzymes[163]. In the latter report, the specific microRNAs linked to the effector CD8$^+$ T cell phenotype were miR-139 and miR-150, which are distinct from the microRNAs identified in our study as regulators of IFN-γ expression, miR-181a-5p and miR-451a.

We found miR-181a-5p to be overexpressed on thymic IFN-γ (YFP$^-$) CD8$^+$ T cells, and could therefore constitute a brake to IFN-γ induction in undifferentiated CD8$^+$ T cells. Consistent with this, miR-181a-5p has Id2 as a biochemically validated target; and we demonstrated that both Id2 downregulation and miR-181a-5p (mimics) transduction reduced IFN-γ mRNA expression in effector CD8$^+$ T cells. Id2 is a key regulator of effector CD8$^+$ T cell differentiation and maintenance *in vivo* [186,187]; and has also been shown to promote the differentiation of IFN-γ-producing CD4$^+$ T (so-called Th1) cells upon viral infection [183]. There is a clear mechanistic link between Id2 and IFN-γ, since Id2 directly antagonizes the suppressive E proteins that bind to regulatory elements of *Tbx21*, which encodes the master transcription factor T-bet [183]. Our results with Id2 knockdown, which very nicely phenocopied those of miR-181a-5p mimics, constituted a critical validation of our experimental approach.

The new function disclosed here for miR-181a-5p in the regulation of IFN-γ production adds to its established role in thymic positive selection, where it modulates TCR signalling towards augmenting thymocyte sensitivity to peptide antigens thus guaranteeing the clonal deletion of moderate-affinity self-reactive clones [192,193]. Interestingly, this known role of miR-181a-5p also involved the modulation of signalling components, which supports this type of mechanism as characteristic of miR-181a-5p function in T cells [192].

As for miR-451a, it had been previously shown to limit CD4$^+$ T cell proliferation *in vitro* and *in vivo* via *Myc* targeting [194]. We now reveal a role for miR-451a in effector CD8$^+$ T cells: interestingly, miR-451a was overexpressed in IFN-γ^+ (YFP$^+$) CD8$^+$ T cells, which suggests a role in fine tuning IFN-γ production in committed effector CD8$^+$ T cells.

From a therapeutic point of view, the manipulation of microRNAs may offer the possibility to modulate IFN-γ responses by human CD8$^+$ T cells, consistent with previous findings where RNA interference-mediated knockdown of Dicer led to an increase in the expression of effector molecules and cytokines such as granzyme B and IFN-γ [163]. Specifically for miR-181a-5p and miR-451a, their therapeutic potential is further supported by reported successful manipulation in human cells [195,196]. We thus believe that

this study adds significantly to our understanding of the post-transcriptional regulation of IFN-γ production by $CD8^+$ T cells, which may have implications for immunity against intracellular pathogens and tumours, as well as for the control of inflammatory conditions.

4.2. - Transcriptional and post-transcriptional regulation of differentiation of γδ T cell subsets

4.2.1 - Introduction

4.2.1.1 - *Development and functions of γδ T cells*

T cells are often divided into conventional or unconventional subgroups. Both develop in the thymus but the first encompasses T cells expressing a TCR composed by an α and a β chain together with co-receptors CD4 or CD8 [197]. They are only able to recognize antigens presented to them by other cells via major histocompatibility complex (MHC) proteins and they mostly participate in adaptive immune responses that typically take several days to be established [197]. The unconventional subgroup, less frequent in secondary lymphoid organs, is composed by cells, such as $\alpha\beta$ NKT cells or $\alpha\beta$ CD8$\alpha\alpha$ intra-epithelial lymphocytes, that expresses $\alpha\beta$ together with other additional surface markers and present distinct homing abilities, or cells whose TCR heterodimer is composed by a γ and a δ chain [198]. These cells are not restricted by MHC presentation; present a limited diversity of TCR rearrangements when compared to the conventional $\alpha\beta$ T cells; often locate to and participate in the homeostasis of specific tissues, that typically constitute a barrier to the outside of the organism such as the skin, gut or other mucosal interfaces; and most importantly, are able to be activated and respond to stimuli, foreign pathogens or transformed cells, in an extremely fast manner, for example by secreting cytokines like IFN-γ or IL-17 that will limit the spreading of the harmful agent and shape the consequent immune response [199]. In order to respond in such a quick manner these cells cannot be, like the conventional T cells, in a naïve state waiting for other cells to provide them signals that will drive their differentiation into a specific effector cell but must already have acquired their functional identity and be on stand by to more swiftly act in response to an infection or stress. In this regard $\gamma\delta$ T cells are extremely interesting since they show a different and unique functional development into mainly two effector phenotypes that are usually defined as IFN-γ producing or IL-17 producing $\gamma\delta$ T cells [200]. They develop in the thymus from lymphoid progenitor cells common to all T cells. During the DN2/DN3 ($CD4^-$ $CD8^-$ double negative) stages thymocytes commit to either the $\alpha\beta$ or the $\gamma\delta$ lineage. After this commitment and during the rest of their thymic development, $\gamma\delta$ T cells can

further differentiate into functionally distinct subgroups by acquiring a specific, and still fairly incompletely characterized, transcriptional program. They are then able to leave the thymus with an established identity and the ability to rapidly respond to stimuli. Nonetheless not all γδ T cells leave the thymus fully mature. They can leave in a naïve state, just like other T cells, and acquire an effector phenotype upon stimulation in the secondary lymphoid organs, blood or other tissues [201]. This functional pre-programming however is not constant over time but occurs in several consecutive waves or time windows during development (Figure 4.2.1) [202]. Interestingly there is a correlation between the wave where a γδ T cell originates from and both the Vγ chain in its TCR and the tissue it migrates to. The first wave, happening exclusively during embryogenesis after embryonic day 13, generates dendritic epidermal γδ T cells (DETCs). These cells that migrate to and populate the epidermis mainly present a TCR with a Vγ5 chain and harbour the ability to produce IFN-γ. In the second wave, also exclusively embryonic, are generated the only γδ T cells that will have the ability to produce IL-17. In addition to lymphoid tissues these cells will be present in the dermis and several mucosal tissues such as the lungs, cornea, tongue and female and male reproductive tracks. They are mostly composed of cells expressing a Vγ6 or Vγ4 TCR, although some Vγ1$^+$ can also be found. Following these two waves that are finished before the animal is born there are three more waves that begin within the embryonic period but continue after birth. γδ NKT cells are born from the third wave, starting at embryonic day 15, and have the potential to produce IFN-γ. The other two functional waves will give rise to intra-epithelial lymphocytes (iIELs), able to produce IFN-γ and often showing a Vγ7 TCR, and to naïve uncommitted γδ T cells that will leave the thymus to the blood, lymphatic vessels and organs [202].

With the ability to rapidly produce the proinflammatory cytokines IFN-γ and IL-17, γδ T cells have shown to play important roles in many of the immune responses where these two cytokines are key mediators. For instance, IFN-γ producing γδ T cells play protective roles in viral infections (e.g. *Influenza*), in bacterial infections (e.g. *Listeria monocytogenes*) and in parasitic infections (e.g. *Plasmodium*) [203–207]. In addition they are also involved in the elimination of tumour cells [66,208]. In turn, IL-17 production by γδ T cells is important in the response against several extracellular bacteria (e.g. *Micobacterium tuberculosis*) and fungi (e.g. *Candida albicans*) [209].

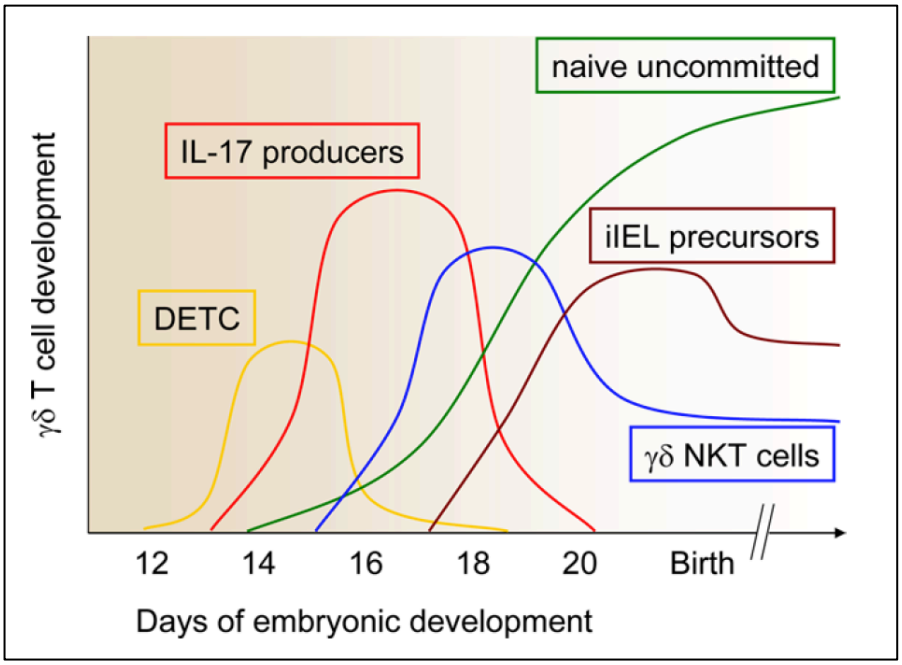


Figure 4.2.1 - Functional development of *γδ* T cells.
This figure illustrates how functionally distinct subsets of *γδ* T cells are generated during different developmental windows. Each functional subset presents a particular and characteristic period during which their cells develop and exit the thymus, happening during the fetal, neonatal and/or adult developmental stages. Figure from Prinz *et al.* 2013 [202].

Due to the great power these two cytokines have, the incorrect control of their expression, in a timely or spatial manner, can lead to situations where their presence, instead of beneficial, becomes detrimental for the organism. Just as how *γδ* T cells are involved in the protective effects these cytokines mediate they can also contribute to the respective damaging effects. They have been shown to play pathogenic roles in promoting several inflammatory and autoimmune diseases such as EAE, psoriasis, uveitis or arthritis and also drive cancer progression in some settings [68,209].

The importance these two *γδ* T cell subsets play in a high number of both protective but also damaging responses shows the relevance of understanding the molecular mechanisms behind their identity establishment and maintenance and also behind their effector abilities. In fact, our lab has previously explored the role of histone epigenetic modification within this context. We found that although CD27$^-$ *γδ* T cells are characterized as being IL-17 producers, the IFN-*γ* locus in these cells is not epigenetically silenced endowing

them the ability to also produce IFN-γ under some conditions. As such CD27$^-$ $\gamma\delta$ T cells are plastic, being able to become IL-17$^+$ IFN-γ^+ (double producers) upon *in vitro* stimulation with IL-1β and IL-23 or under certain immune responses with substantial inflammation *in vivo* [68,203,210–212]. Unlike CD27$^-$, CD27$^+$ $\gamma\delta$ T cells are committed only to IFN-γ production. Cells resort to various mechanisms simultaneously in order to control gene expression and, just like histone modifications, microRNA-mediated post-transcriptional regulation is one of such mechanisms.

4.2.1.2 - *MicroRNA-mediated regulation of effector functions in γδ T cells*

To this day, very few studies have addressed the role of microRNAs in the biology of $\gamma\delta$ T cells.

Haas and colleagues have shown that the expression of miR-133b and miR-206 is co-regulated with the expression of the *Il-17a/f locus*, which is located in the genomic vicinity (<50kb downstream) of the miR-206/133b cluster [213]. This was found to be true for IL-17 producers in both the $\alpha\beta$ and $\gamma\delta$ T cell branch. However, even though co-expressed neighbouring genes are often functionally connected, the overexpression of these microRNAs had no significant consequence on the ability of these cells to produce the IL-17 cytokine [213,214]. The authors suggest they could still be used as a biomarker for identifying IL-17 producing T lymphocytes. This same group has also investigated the role of members of the microRNA-181 family in $\gamma\delta$ T cell development. They had previously showed that this microRNA, very abundant at the double positive (DP) stage of T cell development, is important for a correct sensing of the TCR signal strength. MiR-181a/b-1 deficiency led to a reduction in the responsiveness to TCR signals by DP thymocytes and to an impaired development of $\alpha\beta$ invariant natural killer (iNKT), whose numbers were extensively reduced both in the thymus and the periphery [215]. On the contrary, the absence of miR-181a/b-1 did not impact on the selection, development and differentiation of $\gamma\delta$ T cells (including $\gamma\delta$ NKT and DETCs), that showed no reduction in their thymic or peripheral numbers [216].

A more recent study, from our lab, describes miR-146a as a microRNA with functional implications in $\gamma\delta$ T cell biology. MiR-146a was found highly expressed in peripheral CD27$^-$ $\gamma\delta$ T cells and this expression pattern was found to be imprinted during their

development within the thymus [217]. Experimentally increasing the levels of miR-146a in γδ T cells led to a reduction in the ability of these cells to produce IFN-γ. These facts seem to point to a possible role of miR-146a in actively restraining, at the posttranscriptional level, the expression of IFN-γ in the $CD27^-$ γδ T cell population. In fact, by analysing γδ T cells in miR-146a deficient mice, the absence of the microRNA was found to increase the frequency of $CD27^-$ double producers in a cell intrinsic manner, whilst showing no impact on the IFN-γ expression by their $CD27^+$ counterparts. This was confirmed in a *Listeria monocytogenes* oral infection model where there is an expansion of the $CD27^-$ $IL\text{-}17^+$ IFN-γ^+ population [203]. At day seven post infection the frequency and absolute numbers of these cells were higher in miR-$146a^{-/-}$ mice than in wild type mice, thus demonstrating that, in addition to steady state, miR-146a also regulates $CD27^-$ cell plasticity under pathophysiological conditions. Using a technique called Ago2 RIP-seq it was possible to identify Nod1 as a potential target of miR-146a responsible for the phenotype observed. Consistently with this idea, $Nod1^{-/-}$ mice showed the opposite phenotypes of the ones observed in miR-$146a^{-/-}$ mice, both in the steady state and during *Listeria monocytogenes* infection [217]. The plasticity of $CD27^-$ γδ T cells is thus kept in check, at least in part, by a balance between Nod1, able to increase the plasticity by promoting IFN-γ production, and miR-146a, able to decrease it by reducing Nod1 mRNA levels.

To my knowledge this is so far the only report of a microRNA whose expression has a functional impact in γδ T cell biology. When comparing the present knowledge about microRNA functions in γδ T cells with that in αβ T cells, one finds a tremendous gap. One of the main aims of the present thesis is precisely to close this gap by generating more knowledge on the roles of microRNAs in γδ T cell differentiation.

4.2.2 - Results

4.2.2.1 - *Isolation of γδ T cells expressing IL-17 or IFN-γ*

What are the characteristics and the molecular mechanisms that control the biology of γδ T cells that express IFN-γ or IL-17 has been a question of interest in the field for several years. In fact some studies have already aimed to characterize the gene expression profile of

effector γδ T cells subsets at the messenger RNA level. Our group, also interested in this topic, has looked at the epigenetic control of gene expression and revealed that effector cells, segregated by the CD27 and CCR6 markers, show distinct histone modification profiles that impact on transcription [211]. Here we aim to provide more insight into this question by looking at yet another layer of control of gene expression regulation, the microRNA-mediated post-transcriptional regulation.

There are several reasons that led us to study microRNAs in γδ T cells. First is the powerful impact microRNAs have been shown to have in T cell biology, largely studied in αβ T cells while still very little exploited in γδ T cells. Second is the observation from our lab that mice lacking microRNAs in T cells showed a lower frequency of IL-17 producing γδ T cells, both in the peripheral lymph nodes and in the thymus (unpublished data). We thus decided to address this question and study more in detail, at both the microRNA and mRNA levels, the effector subsets of γδ T cells that express either IL-17 or IFN-γ.

In the studies where effector γδ T cell populations are isolated and analysed, researchers have so far relied on several extracellular markers that although correlating, to different extents, with the potential of γδ T cells to produce either IFN-γ or IL-17, do not allow the isolation of pure populations. We think this approach generates valuable information but has some important caveats. We believe it may lead to the identification of factors that are more related with the extracellular marker used rather than the actual production of the effector cytokines. It can also lead to the masking of other interesting factors that could be specifically enriched only in the cells actually secreting the effector cytokines, but that escape detection because this enrichment is not present across the rest of the population that is also positive for the extracellular marker but that is not actively producing the effector cytokines.

In order to overcome these limitations and be able to study pure populations of IFN-γ or IL-17 producing γδ T cells, we decided to make use of two reporter mouse strains already established [218,219]. In brief, they were both designed relying on the same strategy, which is introducing a DNA fragment with an internal ribosome entry site (IRES) followed by a fluorescent reporter, eYFP for IFN-γ and GFP for IL-17, downstream of the cytokine's

endogenous translational stop codon. This approach generates a bicistronic mRNA where the cytokine and the reporter can be translated independently into separate proteins (schematics in **Figure 4.2.2 a**). In addition, the transcription of this bicistronic mRNA is under the control of the endogenous cytokine promoter and regulatory regions. With the purpose of simultaneously isolating live γδ T cells expressing IFN-γ or IL-17 we crossed these two mouse strains to generate a line containing both reporters, which we designate from here on in as double reporter (DR).

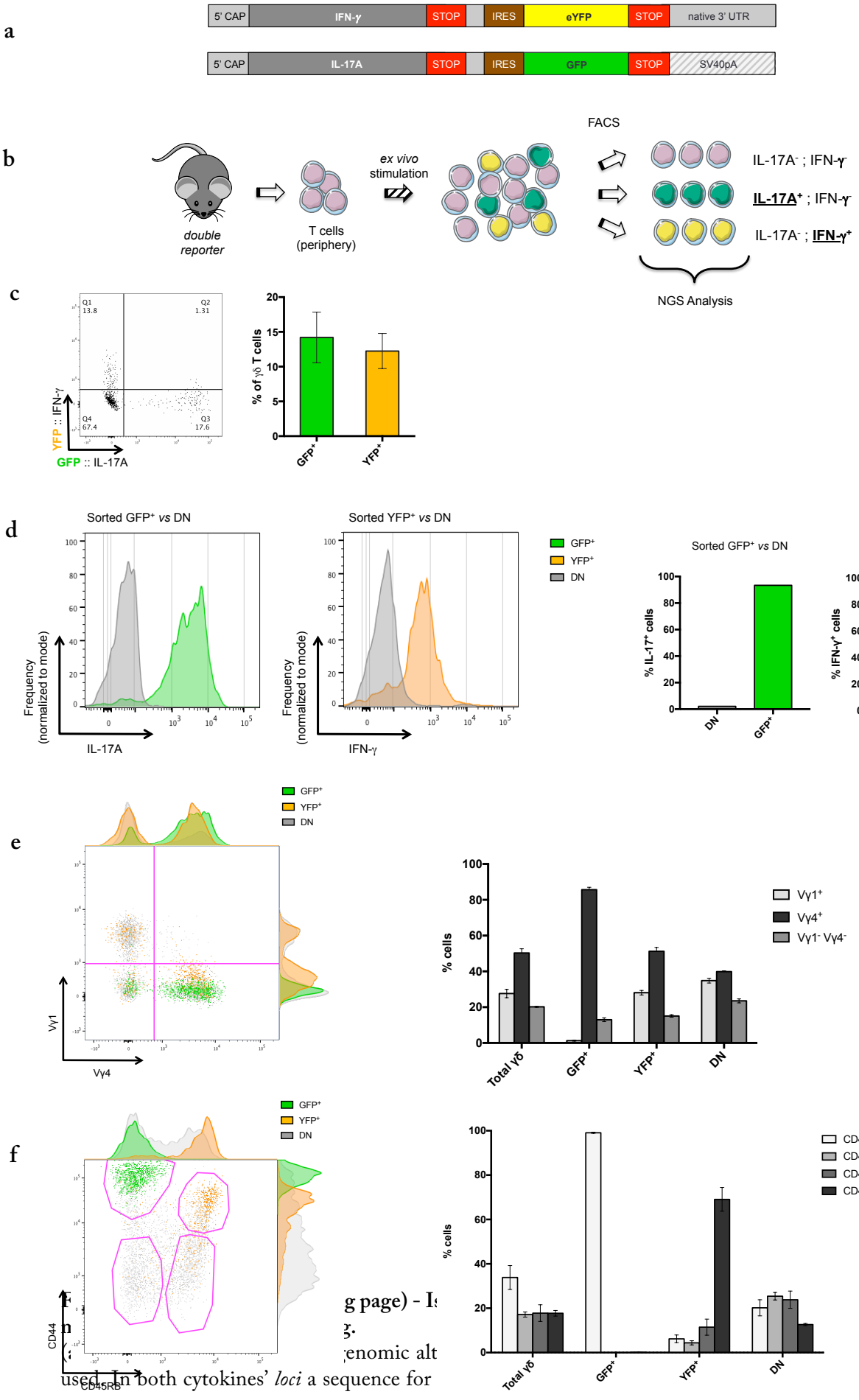
Figure 4.2.2 b illustrates the protocol used to obtain the cells of interest. In short, cells were isolated from the peripheral lymph nodes (PLN) of double reporter animals, stimulated *ex vivo* to allow expression of the cytokines and reporters, and separated into pure populations by fluorescence activated cell sorting (FACS) according to the expression of the YFP and GFP markers (**Figure 4.2.2 c**). Subsequently, total RNA was extracted and two independent libraries were generated for each sample, one for messenger RNA and other for microRNA, and both were submitted to next generation sequencing (NGS).

Intracellular staining for IL-17 and IFN-γ in **Figure 4.2.2 d** confirms that our strategy of sorting cells accordingly to the expression of the two fluorescent reporters, did indeed lead to the obtainment of highly enriched populations of cells expressing either IL-17 (GFP^+) or IFN-γ (YFP^+). We have also characterized the TCR Vγ chain usage amongst these cells (**Figure 4.2.2 e**). GFP^+ cells were mostly $V\gamma4^+$ (~85%) but also presented some $V\gamma1^-$ $V\gamma4^-$ (~15%) which are most likely to be $V\gamma6^+$ cells. On the other hand, YFP^+ cells showed a more balanced distribution, but still with $V\gamma4^+$ as being the most frequent (~50%) followed by $V\gamma1^+$ (~30%) and finally $V\gamma1^-$ $V\gamma4^-$ (~15%). **Figure 4.2.2 f** shows another extracellular characterization but using different markers, CD44 and CD45RB, commonly used to infer the ability of γδ T cells to produce IL-17 or IFN-γ [220]. As expected for cells producing IL-17, GFP^+ cells were exclusively $CD44^{hi}$ $CD45RB^{lo}$. YFP^+ cells were mostly (~70%) $CD44^{hi}$ $CD45RB^{hi}$, described to be phenotype for more mature IFN-γ producing γδ T cells.

4.2.2.2 - *Messenger RNA profile in effector γδ T cell subsets using Next Generation Sequencing*

In the analysis of the data obtained by next generation sequencing we found 7882 genes to be differentially expressed between the GFP^+ and YFP^+ populations (after removing all genes with false discovery rate (FDR) higher than 0.05). From this total, 4341 genes were more expressed in the GFP^+ population and 3541 gene more expressed in the comparing YFP^+ population. Principal component analysis (PCA) of the data revealed that all 3 replicates clustered tightly together and that all 3 populations analysed here: GFP^+, YFP^+ and GFP^- YFP^- (hereafter designated as double negative or DN) were clearly distinct from each other; which increases our confidence in the data and gives us confirmation that we undoubtedly isolated distinct cell populations (**Figure 4.2.3**).

Not surprisingly, and in line with the reasons why we decided to use a reporter system, we found the cytokines IL-17 and IFN-γ amongst the top 5 genes with the highest fold change (**Figure 4.2.4**). In GFP^+ there was 4419 times more IL-17A mRNA present than in YFP^+ cells and, on the other hand, IFN-γ mRNA that was roughly 700 times more expressed in YFP^+ cells than in GFP^+ cells. **Figure 4.2.4 a** shows the top 50 genes with the highest fold change amongst those genes detected to be significantly more expressed in IL-17 producing cells. Similarly, **Figure 4.2.4 b** shows the top 50 genes with the highest fold change but from within the genes more expressed in IFN-γ producing cells.



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used. In both cytokines' *loci* a sequence for

has been inserted downstream of the cytokine's endogenous translational stop codon. (**b**) Graphical overview of the protocol used to isolate the populations of interest. In short, cells were isolated from peripheral lymph nodes of double reporter mice, stimulated with PMA plus ionomycin and separated by FACS accordingly to their fluorescence profile. (**c**) Average proportion of GFP$^+$ and YFP$^+$ $\gamma\delta$ T cells obtained during FACS sessions (right) with a representative plot (left). (**d**) Intracellular staining for IL-17 and IFN-γ performed on sorted $\gamma\delta$ T cell populations. (**e**) Characterization of the Vγ chain usage, determined by Vγ1 and Vγ4 staining, within the GFP$^+$, YFP$^+$ and DN populations. (**f**) Characterization of CD44 and CD45RB extracellular markers within the GFP$^+$, YFP$^+$ and DN populations.

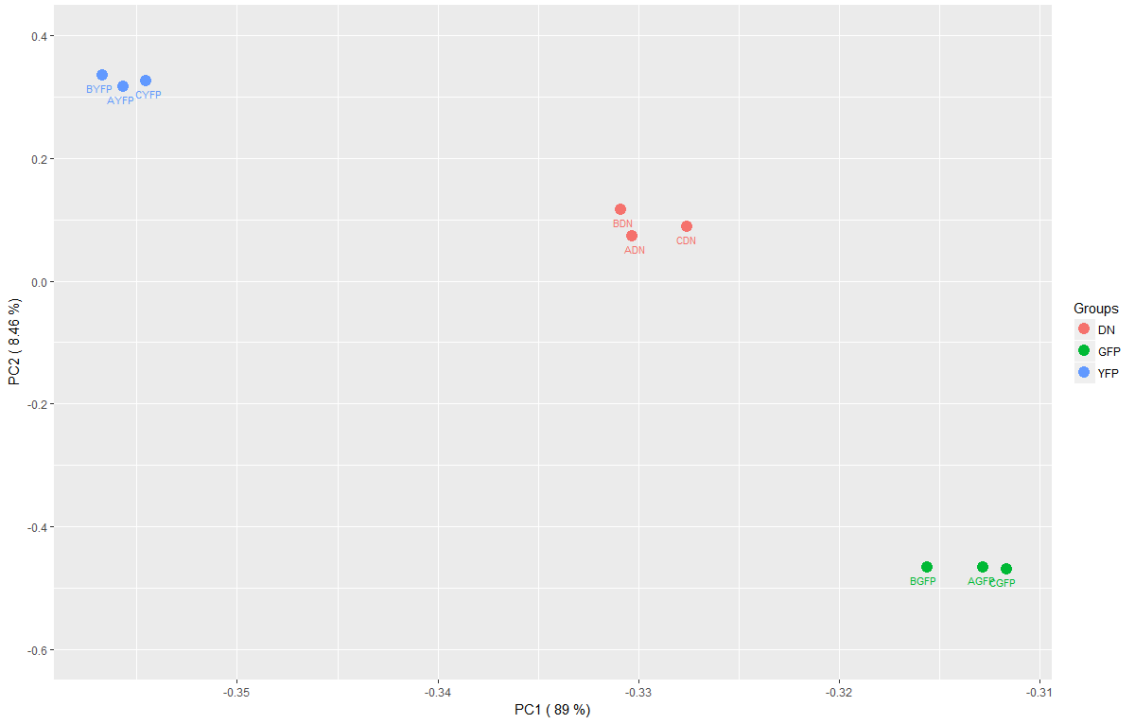


Figure 4.2.3 - Principal component analysis (PCA) plot from messenger RNA sequencing data analysis.
PCA of all the 9 samples used for messenger RNA sequencing. Each population of cells isolated: GFP$^+$ YFP$^-$ (labelled GFP, in green); GFP$^-$ YFP$^+$ (labelled YFP, in blue) and GFP$^-$ YFP$^-$ (labelled DN, in red) is represented by its 3 independent samples labelled A, B and C. All 3 samples from the same cellular population cluster tightly together and distantly from the other 2 populations.

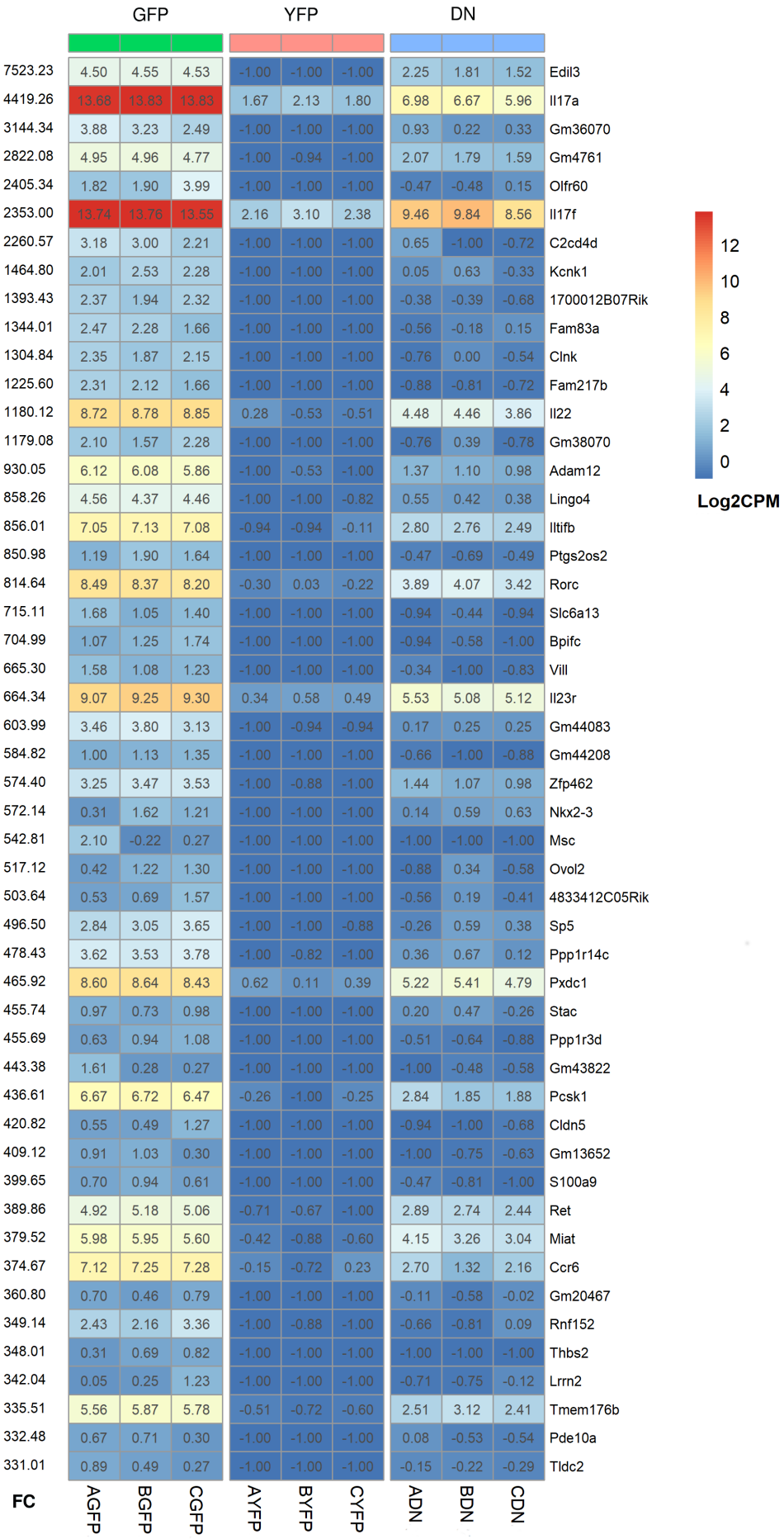
Together with the IL-17A and IFN-γ genes and amid the very top 50 of the most differently expressed genes (out of a total of 4341 and 3541 genes respectively) we found several genes whose expression was expected to be associated with one of the two effector subsets. For example, together with IL-17A we found highly enriched transcripts for other

cytokines like *Il17f* (FC=2353) and *Il22* (FC=1180), transcription factors as *Rorc* (FC=815) and some membrane receptors such as *Il23r* (FC=664) *Ccr6* (FC=375). We confirmed some of these results, namely the cytokines and their respective reporters (GFP our YFP), used to sort the cells, on samples collected independently by performing quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) (**Figure 4.2.5**).

However there are many other genes, some coding for extracellular receptors for example, that have yet to be associated with either one these effector profiles of *γδ* T cells. Some are known genes within the T cell biology but others, unexpectedly are not and can most definitely constitute the basis for very interesting future studies to unravel their role in *γδ* T cell biology.

Further filtering of the 7882 genes list to remove those presenting a fold change lower than 1.5 revealed 6809 genes differently expressed between the two populations. 3885 had a higher expression in GFP^+ cells and 2924 genes a higher expression in YFP^+ cells. We then used this more restrict gene set to look for particular genes of interest and to gain more information on which pathways and processes are enriched when comparing IL-17 producing *versus* IFN-*γ* producing *γδ* T cells.

a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3144.34 3.88 3.23 2.49 -1.00 -1.00 -1.00 0.93 0.22 0.33 Gm36070
2822.08 4.95 4.96 4.77 -1.00 -0.94 -1.00 2.07 1.79 1.59 Gm4761
2405.34 1.82 1.90 3.99 -1.00 -1.00 -1.00 -0.47 -0.48 0.15 Olfr60
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1464.80 2.01 2.53 2.28 -1.00 -1.00 -1.00 0.05 0.63 -0.33 Kcnk1
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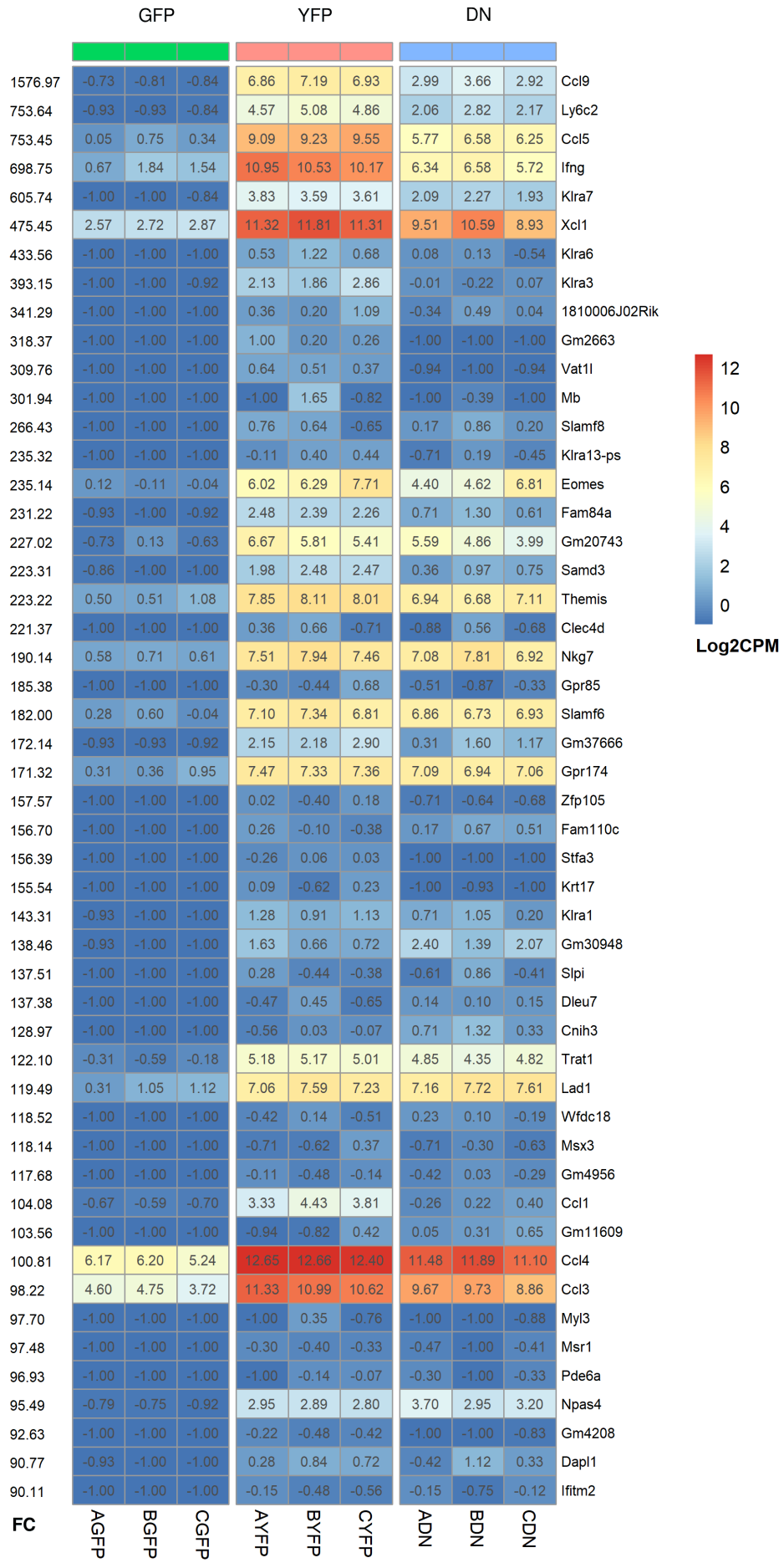
GFP
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DN
1576.97 -0.73 -0.81 -0.84 6.86 7.19 6.93 2.99 3.66 2.92 Ccl9
753.64 -0.93 -0.93 -0.84 4.57 5.08 4.86 2.06 2.82 2.17 Ly6c2
753.45 0.05 0.75 0.34 9.09 9.23 9.55 5.77 6.58 6.25 Ccl5
698.75 0.67 1.84 1.54 10.95 10.53 10.17 6.34 6.58 5.72 Ifng
605.74 -1.00 -1.00 -0.84 3.83 3.59 3.61 2.09 2.27 1.93 Klra7
475.45 2.57 2.72 2.87 11.32 11.81 11.31 9.51 10.59 8.93 Xcl1
433.56 -1.00 -1.00 -1.00 0.53 1.22 0.68 0.08 0.13 -0.54 Klra6
393.15 -1.00 -1.00 -0.92 2.13 1.86 2.86 -0.01 -0.22 0.07 Klra3
341.29 -1.00 -1.00 -1.00 0.36 0.20 1.09 -0.34 0.49 0.04 1810006J02Rik
318.37 -1.00 -1.00 -1.00 1.00 0.20 0.26 -1.00 -1.00 -1.00 Gm2663
309.76 -1.00 -1.00 -1.00 0.64 0.51 0.37 -0.94 -1.00 -0.94 Vat1l
301.94 -1.00 -1.00 -1.00 -1.00 1.65 -0.82 -1.00 -0.39 -1.00 Mb
266.43 -1.00 -1.00 -1.00 0.76 0.64 -0.65 0.17 0.86 0.20 Slamf8
235.32 -1.00 -1.00 -1.00 -0.11 0.40 0.44 -0.71 0.19 -0.45 Klra13-ps
235.14 0.12 -0.11 -0.04 6.02 6.29 7.71 4.40 4.62 6.81 Eomes
231.22 -0.93 -1.00 -0.92 2.48 2.39 2.26 0.71 1.30 0.61 Fam84a
227.02 -0.73 0.13 -0.63 6.67 5.81 5.41 5.59 4.86 3.99 Gm20743
223.31 -0.86 -1.00 -1.00 1.98 2.48 2.47 0.36 0.97 0.75 Samd3
223.22 0.50 0.51 1.08 7.85 8.11 8.01 6.94 6.68 7.11 Themis
221.37 -1.00 -1.00 -1.00 0.36 0.66 -0.71 -0.88 0.56 -0.68 Clec4d
190.14 0.58 0.71 0.61 7.51 7.94 7.46 7.08 7.81 6.92 Nkg7
185.38 -1.00 -1.00 -1.00 -0.30 -0.44 0.68 -0.51 -0.87 -0.33 Gpr85
182.00 0.28 0.60 -0.04 7.10 7.34 6.81 6.86 6.73 6.93 Slamf6
172.14 -0.93 -0.93 -0.92 2.15 2.18 2.90 0.31 1.60 1.17 Gm37666
171.32 0.31 0.36 0.95 7.47 7.33 7.36 7.09 6.94 7.06 Gpr174
157.57 -1.00 -1.00 -1.00 0.02 -0.40 0.18 -0.71 -0.64 -0.68 Zfp105
156.70 -1.00 -1.00 -1.00 0.26 -0.10 -0.38 0.17 0.67 0.51 Fam110c
156.39 -1.00 -1.00 -1.00 -0.26 0.06 0.03 -1.00 -1.00 -1.00 Stfa3
155.54 -1.00 -1.00 -1.00 0.09 -0.62 0.23 -1.00 -0.93 -1.00 Krt17
143.31 -0.93 -1.00 -1.00 1.28 0.91 1.13 0.71 1.05 0.20 Klra1
138.46 -0.93 -1.00 -1.00 1.63 0.66 0.72 2.40 1.39 2.07 Gm30948
137.51 -1.00 -1.00 -1.00 0.28 -0.44 -0.38 -0.61 0.86 -0.41 Slpi
137.38 -1.00 -1.00 -1.00 -0.47 0.45 -0.65 0.14 0.10 0.15 Dleu7
128.97 -1.00 -1.00 -1.00 -0.56 0.03 -0.07 0.71 1.32 0.33 Cnih3
122.10 -0.31 -0.59 -0.18 5.18 5.17 5.01 4.85 4.35 4.82 Trat1
119.49 0.31 1.05 1.12 7.06 7.59 7.23 7.16 7.72 7.61 Lad1
118.52 -1.00 -1.00 -1.00 -0.42 0.14 -0.51 0.23 0.10 -0.19 Wfdc18
118.14 -1.00 -1.00 -1.00 -0.71 -0.62 0.37 -0.71 -0.30 -0.63 Msx3
117.68 -1.00 -1.00 -1.00 -0.11 -0.48 -0.14 -0.42 0.03 -0.29 Gm4956
104.08 -0.67 -0.59 -0.70 3.33 4.43 3.81 -0.26 0.22 0.40 Ccl1
103.56 -1.00 -1.00 -1.00 -0.94 -0.82 0.42 0.05 0.31 0.65 Gm11609
100.81 6.17 6.20 5.24 12.65 12.66 12.40 11.48 11.89 11.10 Ccl4
98.22 4.60 4.75 3.72 11.33 10.99 10.62 9.67 9.73 8.86 Ccl3
97.70 -1.00 -1.00 -1.00 -1.00 0.35 -0.76 -1.00 -1.00 -0.88 Myl3
97.48 -1.00 -1.00 -1.00 -0.30 -0.40 -0.33 -0.47 -1.00 -0.41 Msr1
96.93 -1.00 -1.00 -1.00 -1.00 -0.14 -0.07 -0.30 -1.00 -0.33 Pde6a
95.49 -0.79 -0.75 -0.92 2.95 2.89 2.80 3.70 2.95 3.20 Npas4
92.63 -1.00 -1.00 -1.00 -0.22 -0.48 -0.42 -1.00 -1.00 -0.83 Gm4208
90.77 -0.93 -1.00 -1.00 0.28 0.84 0.72 -0.42 1.12 0.33 Dapl1
90.11 -1.00 -1.00 -1.00 -0.15 -0.48 -0.56 -0.15 -0.75 -0.12 Ifitm2
FC
AGFP BGFP CGFP AYFP BYFP CYFP ADN BDN CDN
12
10
8
6
4
2
0
Log2CPM

Figure 4.2.4 (shown on preceding pages) - Top differentially expressed genes between GFP⁺ and YFP⁺ γδ T cells with a fold FDR ≤ 0.05.
(**a**) Top 50 genes found more expressed in GFP⁺ cells. (**b**) Top 50 genes found more expressed in YFP⁺ cells. In both (**a-b**), genes are ordered by decreasing fold change value (FC). FC is indicated on the left side and gene names are indicated on the right side. Values inside rectangles refer to the individual log_2CPM (Counts Per Million) values of each replicate (named A, B and C) for each sample (GFP for GFP⁺; YFP for YFP⁺ and DN for double negative GFP⁻ YFP⁻).

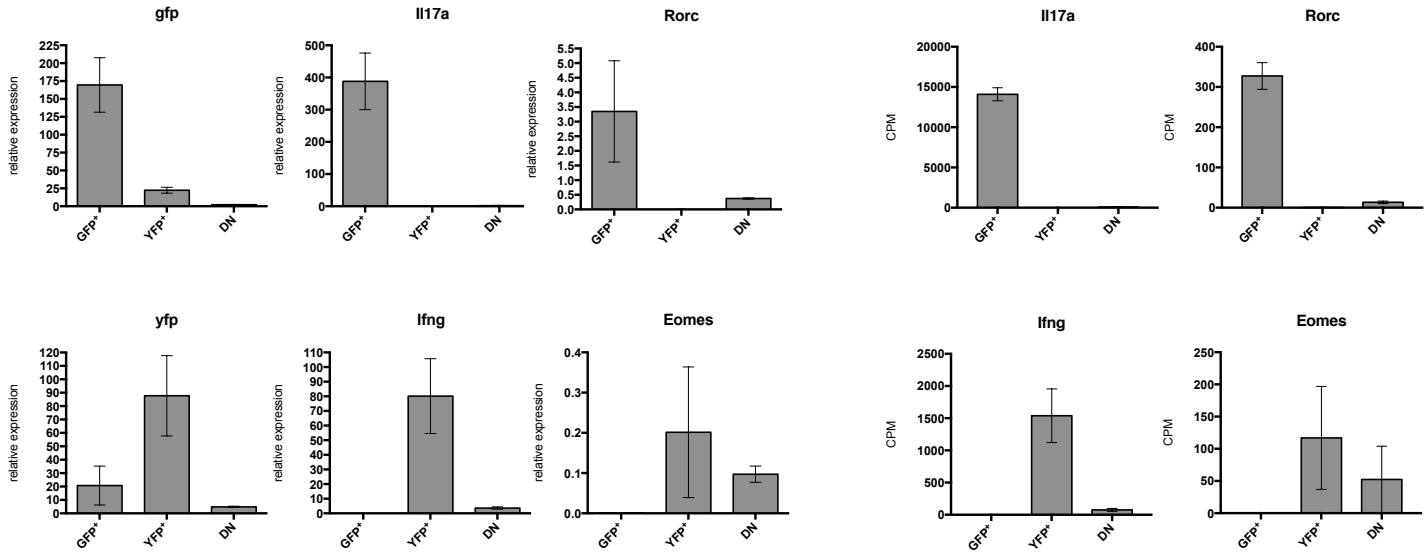


Figure 4.2.5 - RT-qPCR confirmation of cytokine and reporter gene expression in GFP⁺ and YFP⁺ γδ T cells.
RT-qPCR on samples collected independently (6 graphs on the left side) confirmed the results obtained by NGS (4 graphs on the right side). *Il17a* correlated with and was specific and highly enriched in GFP⁺ cells, where *Rorc* was also enriched (top row). YFP⁺ cells were the only population with high levels of *Ifng*, and where we could also find the highest *Eomes* expression.

4.2.2.3 - *Gene Ontology (GO) term enrichment analysis*

The two lists of genes enriched in either effector phenotype with a FC≥1.5 were submitted to gene ontology subsets enrichment analysis (GO Slim) in order to retrieve the most common or significantly over-represented GO terms associated with the listed genes in the categories of the GO system of classification, namely biological process (**Figure 4.2.6**), molecular function (**Figure 4.2.7**) and cellular component (**Figure 4.2.8**).

There were many biological processes detected as significantly represented in each population of cells, more specifically 36 for GFP$^+$ and 31 for YFP$^+$ cells (**Figure 4.2.6**). In IL-17 producing *γδ* T cells the processes showing the lowest p-value are: transport, response to stress, vesicle-mediated transport and cell motility. In this list we could also find other very interesting processes such as signal transduction, with 926 genes present belonging to this category, vacuolar transport, cell death, autophagy, and finally, metabolic related classes such as catabolic process, lipid metabolic process and carbohydrate metabolic process (**Figure 4.2.6 a**). IFN-*γ* expressing *γδ* T cells seemed to be enriched in several processes related with RNA and DNA biology including translation and ribosome biogenesis, mRNA processing, nucleic acid metabolism and cell division along with other diverse processes (**Figure 4.2.6 b**).

In what refers to molecular function we found 6 GO Slim classification classes for IL-17 expressing *γδ* T cells and 10 for IFN-*γ* expressing *γδ* T cells (**Figure 4.2.7**). This later ones include several implicated in RNA and ribosomal biology such as RNA binding, structural constituent of ribosome, mRNA binding, rRNA binding and unfolded protein binding (**Figure 4.2.7 b**). Other molecular functions found include structural molecule activity, methyltransferase activity, isomerase activity, ligase activity and protein transporter activity. In IL-17 producers the molecular functions identified are: enzyme binding, ion binding and cytoskeletal binding in addition to and enzyme regulator activity, kinase activity and GTPase activity (**Figure 4.2.7 a**). These later functions are likely to be related to processes such as signalling pathways.

When looking at which cellular components the genes enriched in each population associate with we also found some interesting differences (**Figure 4.2.8**). In IL-17$^+$ cells we have as unique terms: plasma membrane, cytoplasmic vesicle, endoplasmic reticulum, Golgi apparatus, endosome, vacuole, lysosome and also nuclear envelope (**Figure 4.2.8 a**). From the 3885 genes over 800 are annotated to associate with the plasma membrane and a considerate portion of this is likely to be some kind of receptor for cytokines, chemokines or other signalling molecules. In IFN-*γ*$^+$ we found several categories that relate with the nucleus and chromatin dynamics, for example nucleus, nucleoplasm, nucleolus, chromosome and nuclear chromosome (**Figure 4.2.8 b**). Ribosome was also present in our

list and is an interesting observation as it is likely correlated with the nucleolus and protein-containing complex GO terms also present, likely all contributing to an efficient translation of coding transcripts. Intriguingly over 400 genes, out of almost 3000 in total, are related to processes that take place at the mitochondrion. Finally, microtubule-organizing centre (MTOC) has just short of 100 related genes in the YFP^+ list, and might be related with a different ability of these cells in conducting cell division, interacting with antigen presenting cells or even migration [221].

Differences between the populations of IL-17 producing and IFN-γ producing $\gamma\delta$ T cells were found in all three categories of the GO Slim system of classification: biological process, molecular function and cellular component. From this analysis we can already observe that there are fundamental differences between the two populations and start to gain some insight into the distinct aspects of these cells' biology.

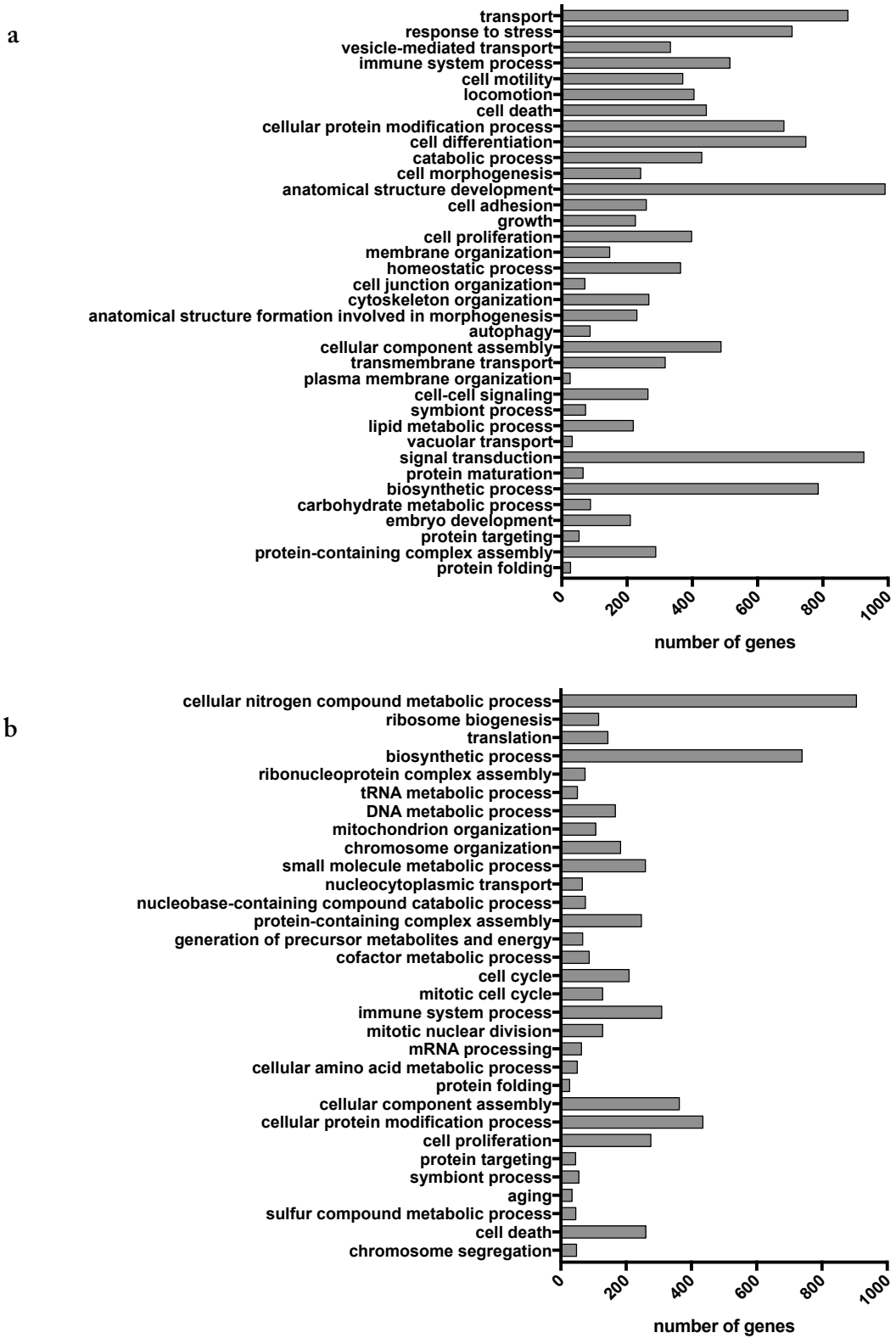
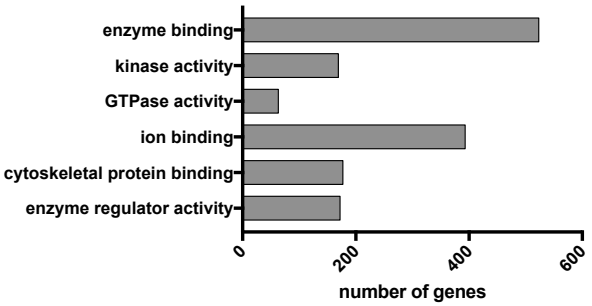


Figure 4.2.6 - Biological Processes enriched in GFP⁺ *vs* YFP⁺ *γδ* T cells.
Number of genes for each of the most represented biological processes (GO Slim) in GFP⁺ IL-17 expressing (**a**) and in YFP⁺ IFN-*γ* expressing (**b**) *γδ* T cells. GO terms are ordered from top to bottom according to increasing p-value.

a



b

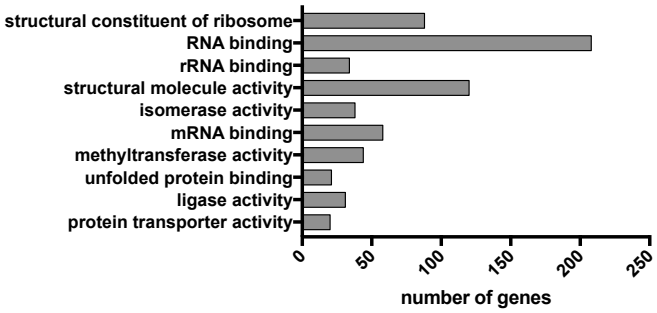
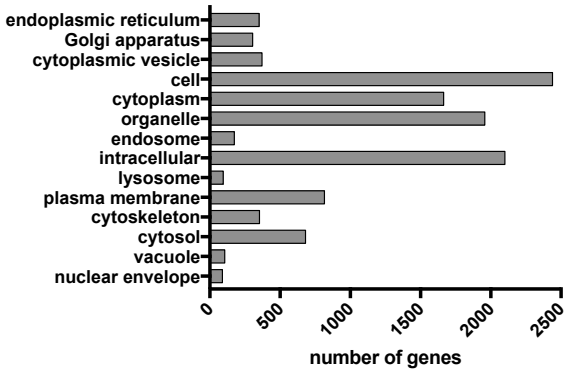


Figure 4.2.7 - Molecular Functions enriched in GFP⁺ *vs* YFP⁺ *γδ* T cells.
Number of genes for each of the most represented molecular functions (GO Slim) of the genes enriched in GFP⁺ IL-17 expressing (**a**) and in YFP⁺ IFN-*γ* expressing (**b**) *γδ* T cells. GO terms are ordered from top to bottom according to increasing p-value.

a



b

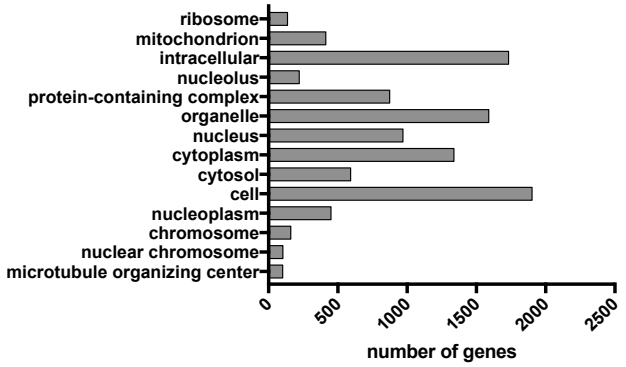


Figure 4.2.8 - Cellular Components enriched in GFP⁺ *vs* YFP⁺ *γδ* T cells.
Number of genes for each of the most represented cellular components (GO Slim) to which the genes enriched in either GFP⁺ IL-17 expressing (**a**) or YFP⁺ IFN-*γ* expressing (**b**) *γδ* T cells are associated with. GO terms are ordered from top to bottom according to increasing p-value.

4.2.2.4 - *Pathway enrichment analysis*

Gene Ontology analysis is a good starting point for understanding what are the molecular processes that are unique to and responsible for the different abilities and characteristics of the two effector subsets under study. However, we aimed at a deeper and more complete view of these processes by asking, based on the transcriptomic data obtained, which pathways were more likely to be relevant for the biology of each of the subsets. For that we resorted to the KEGG database and tested for pathways enriched in the GFP^+ or YFP^+ cells when compared to each other.

A total of 106 KEGG pathways were significantly represented in GFP^+ cells. After removing those related with diseases we obtained a total of 62 pathways (**Table 4.5**), whose KEGG classes are summarized in **Table 4.1**. The class with the most number of pathways represented is the Environmental Information Processing with 17 pathways involved in signal transduction and 3 more involved in signalling molecules and interaction. It is interesting to note that the next classes with the most number of pathways present are all under Organismal Systems and do not only include the immune system, as it was to be expected, but also an unexpected endocrine and nervous system. There are also several pathways that fall within the general metabolism class and more specifically into carbohydrate metabolism, lipid metabolism, glycan biosynthesis and metabolism. All of these are different from the ones that were detected in the YFP^+ cells. These cells, that express IFN-γ, in turn showed 39 enriched pathways that turned into 27 once we removed pathways related with diseases (**Table 4.6**) and whose classes are summarized in **Table 4.2**. As it was already reflected in the GO Slim analysis, these cells present several pathways involved in processes related with DNA or RNA, namely replication and repair, transcription and translation. Several metabolic subclasses were also detected that differ from the ones in IL-17 producers, namely the metabolism of nucleotides, amino acids, carbohydrates, cofactors and enzymes and also energy metabolism.

Although computationally inferred, results from analysis of the same data using the Reactome pathway database are also very informative because, in addition to matching with the GO Slim and KEGG pathway analysis presented above, they provide additional

information since the pathway classification is not as broad and evaluates more specific pathways (**Table 4.7** and **Table 4.8**). Using this database, GFP$^+$ cells showed 93 significantly represented pathways (opposed to 63 using KEGG) and YFP$^+$ cells notably displayed 203 pathways (opposed to 27 using KEGG).

The hypotheses raised in the discussion were supported and elaborated based on these data. These results reveal that there are substantial differences between an IFN-γ producing versus an IL-17 producing $\gamma\delta$ T cell that go beyond their abilities to produce different cytokines and extend to fundamental cellular processes, such as metabolism, or more specialized ones, such as integration of extracellular signals.

Table 4.1 - KEGG classes of the pathways found enriched in GFP$^+$ $\gamma\delta$ T cells.

KEGG Class	*Number of KEGG pathways*
Environmental Information Processing; Signal transduction	17
Organismal Systems; Immune system	13
Organismal Systems; Endocrine system	6
Organismal Systems; Nervous system	5
Cellular Processes; Cell growth and death	3
Cellular Processes; Cellular community - eukaryotes	3
Cellular Processes; Transport and catabolism	3
Environmental Information Processing; Signalling molecules and interaction	3
Metabolism; Carbohydrate metabolism	2
Metabolism; Lipid metabolism	2
Organismal Systems; Development	2
Cellular Processes; Cell motility	1
Genetic Information Processing; Folding, sorting and degradation	1
Metabolism; Glycan biosynthesis and metabolism	1

Note: The number of KEGG pathways detected per KEGG class is indicated on the right side.

Table 4.2 - KEGG classes of the pathways found enriched in YFP⁺ *γδ* T cells.

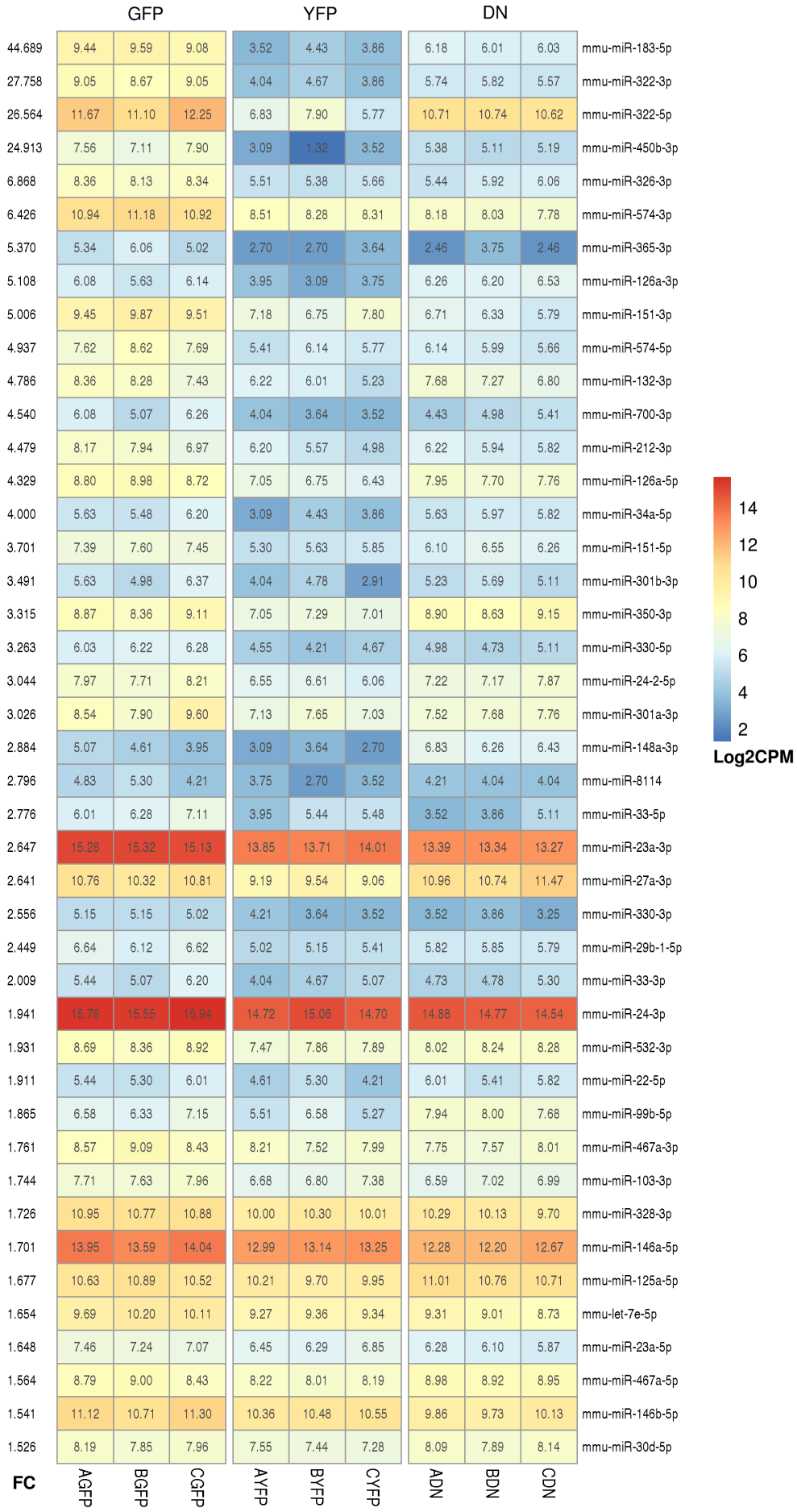
KEGG Class	*Number of KEGG pathways*
Organismal Systems; Immune system	6
Genetic Information Processing; Replication and repair	4
Genetic Information Processing; Translation	3
Genetic Information Processing; Transcription	2
Genetic Information Processing; Folding, sorting and degradation	2
Metabolism; Nucleotide metabolism	2
Metabolism; Amino acid metabolism	1
Metabolism; Carbohydrate metabolism	1
Metabolism; Metabolism of cofactors and vitamins	1
Metabolism; Energy metabolism	1
Cellular Processes; Cell growth and death	1
Metabolic pathways	-
Carbon Metabolism	-
Biosynthesis of amino acids	-

Note: The number of KEGG pathways detected per KEGG class is indicated on the right side.

4.2.2.5 - *MicroRNA profiling of effector γδ T cell subsets using Next Generation Sequencing*

Profiling of the miRNome of GFP⁺ and YFP⁺ *γδ* T cells revealed 103 mature microRNAs with significant differential expression between these two populations. A total of 43 microRNAs were enriched in the population expressing IL-17 (GFP⁺) while the remaining 60 were more expressed in the cells producing IFN-*γ* (YFP⁺). **Figure 4.2.9 a** shows all microRNAs differentially expressed and enriched in GFP⁺ while **Figure 4.2.9 b** shows the top 50 microRNAs presenting the biggest enrichment in YFP⁺ *γδ* T cells. Just like in the transcriptomic data analysis, principal component analysis revealed that samples cluster into 3 distinct populations, which all matched correctly with their effector profile (**Figure 4.2.10**).

a



FC	AGFP	BGFP	CGFP	AYFP	BYFP	CYFP	ADN	BDN	CDN	
44.689	9.44	9.59	9.08	3.52	4.43	3.86	6.18	6.01	6.03	mmu-miR-183-5p
27.758	9.05	8.67	9.05	4.04	4.67	3.86	5.74	5.82	5.57	mmu-miR-322-3p
26.564	11.67	11.10	12.25	6.83	7.90	5.77	10.71	10.74	10.62	mmu-miR-322-5p
24.913	7.56	7.11	7.90	3.09	1.32	3.52	5.38	5.11	5.19	mmu-miR-450b-3p
6.868	8.36	8.13	8.34	5.51	5.38	5.66	5.44	5.92	6.06	mmu-miR-326-3p
6.426	10.94	11.18	10.92	8.51	8.28	8.31	8.18	8.03	7.78	mmu-miR-574-3p
5.370	5.34	6.06	5.02	2.70	2.70	3.64	2.46	3.75	2.46	mmu-miR-365-3p
5.108	6.08	5.63	6.14	3.95	3.09	3.75	6.26	6.20	6.53	mmu-miR-126a-3p
5.006	9.45	9.87	9.51	7.18	6.75	7.80	6.71	6.33	5.79	mmu-miR-151-3p
4.937	7.62	8.62	7.69	5.41	6.14	5.77	6.14	5.99	5.66	mmu-miR-574-5p
4.786	8.36	8.28	7.43	6.22	6.01	5.23	7.68	7.27	6.80	mmu-miR-132-3p
4.540	6.08	5.07	6.26	4.04	3.64	3.52	4.43	4.98	5.41	mmu-miR-700-3p
4.479	8.17	7.94	6.97	6.20	5.57	4.98	6.22	5.94	5.82	mmu-miR-212-3p
4.329	8.80	8.98	8.72	7.05	6.75	6.43	7.95	7.70	7.76	mmu-miR-126a-5p
4.000	5.63	5.48	6.20	3.09	4.43	3.86	5.63	5.97	5.82	mmu-miR-34a-5p
3.701	7.39	7.60	7.45	5.30	5.63	5.85	6.10	6.55	6.26	mmu-miR-151-5p
3.491	5.63	4.98	6.37	4.04	4.78	2.91	5.23	5.69	5.11	mmu-miR-301b-3p
3.315	8.87	8.36	9.11	7.05	7.29	7.01	8.90	8.63	9.15	mmu-miR-350-3p
3.263	6.03	6.22	6.28	4.55	4.21	4.67	4.98	4.73	5.11	mmu-miR-330-5p
3.044	7.97	7.71	8.21	6.55	6.61	6.06	7.22	7.17	7.87	mmu-miR-24-2-5p
3.026	8.54	7.90	9.60	7.13	7.65	7.03	7.52	7.68	7.76	mmu-miR-301a-3p
2.884	5.07	4.61	3.95	3.09	3.64	2.70	6.83	6.26	6.43	mmu-miR-148a-3p
2.796	4.83	5.30	4.21	3.75	2.70	3.52	4.21	4.04	4.04	mmu-miR-8114
2.776	6.01	6.28	7.11	3.95	5.44	5.48	3.52	3.86	5.11	mmu-miR-33-5p
2.647	15.28	15.32	15.13	13.85	13.71	14.01	13.39	13.34	13.27	mmu-miR-23a-3p
2.641	10.76	10.32	10.81	9.19	9.54	9.06	10.96	10.74	11.47	mmu-miR-27a-3p
2.556	5.15	5.15	5.02	4.21	3.64	3.52	3.52	3.86	3.25	mmu-miR-330-3p
2.449	6.64	6.12	6.62	5.02	5.15	5.41	5.82	5.85	5.79	mmu-miR-29b-1-5p
2.009	5.44	5.07	6.20	4.04	4.67	5.07	4.73	4.78	5.30	mmu-miR-33-3p
1.941	15.78	15.55	15.94	14.72	15.06	14.70	14.88	14.77	14.54	mmu-miR-24-3p
1.931	8.69	8.36	8.92	7.47	7.86	7.89	8.02	8.24	8.28	mmu-miR-532-3p
1.911	5.44	5.30	6.01	4.61	5.30	4.21	6.01	5.41	5.82	mmu-miR-22-5p
1.865	6.58	6.33	7.15	5.51	6.58	5.27	7.94	8.00	7.68	mmu-miR-99b-5p
1.761	8.57	9.09	8.43	8.21	7.52	7.99	7.75	7.57	8.01	mmu-miR-467a-3p
1.744	7.71	7.63	7.96	6.68	6.80	7.38	6.59	7.02	6.99	mmu-miR-103-3p
1.726	10.95	10.77	10.88	10.00	10.30	10.01	10.29	10.13	9.70	mmu-miR-328-3p
1.701	13.95	13.59	14.04	12.99	13.14	13.25	12.28	12.20	12.67	mmu-miR-146a-5p
1.677	10.63	10.89	10.52	10.21	9.70	9.95	11.01	10.76	10.71	mmu-miR-125a-5p
1.654	9.69	10.20	10.11	9.27	9.36	9.34	9.31	9.01	8.73	mmu-let-7e-5p
1.648	7.46	7.24	7.07	6.45	6.29	6.85	6.28	6.10	5.87	mmu-miR-23a-5p
1.564	8.79	9.00	8.43	8.22	8.01	8.19	8.98	8.92	8.95	mmu-miR-467a-5p
1.541	11.12	10.71	11.30	10.36	10.48	10.55	9.86	9.73	10.13	mmu-miR-146b-5p
1.526	8.19	7.85	7.96	7.55	7.44	7.28	8.09	7.89	8.14	mmu-miR-30d-5p

b

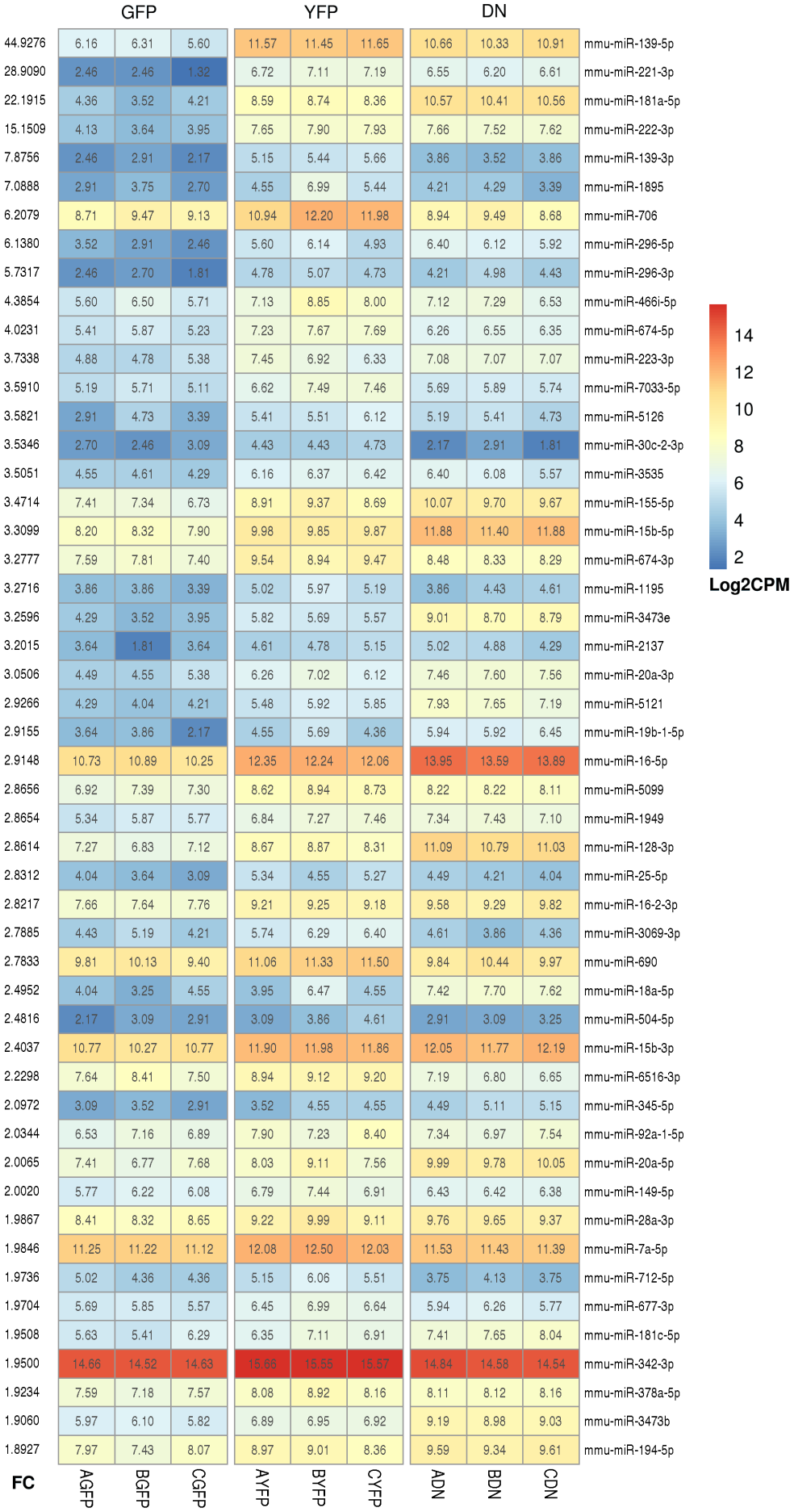

GFP
YFP
DN
FC
AGFP BGFP CGFP AYFP BYFP CYFP ADN BDN CDN
44.9276 6.16 6.31 5.60 11.57 11.45 11.65 10.66 10.33 10.91 mmu-miR-139-5p
28.9090 2.46 2.46 1.32 6.72 7.11 7.19 6.55 6.20 6.61 mmu-miR-221-3p
22.1915 4.36 3.52 4.21 8.59 8.74 8.36 10.57 10.41 10.56 mmu-miR-181a-5p
15.1509 4.13 3.64 3.95 7.65 7.90 7.93 7.66 7.52 7.62 mmu-miR-222-3p
7.8756 2.46 2.91 2.17 5.15 5.44 5.66 3.86 3.52 3.86 mmu-miR-139-3p
7.0888 2.91 3.75 2.70 4.55 6.99 5.44 4.21 4.29 3.39 mmu-miR-1895
6.2079 8.71 9.47 9.13 10.94 12.20 11.98 8.94 9.49 8.68 mmu-miR-706
6.1380 3.52 2.91 2.46 5.60 6.14 4.93 6.40 6.12 5.92 mmu-miR-296-5p
5.7317 2.46 2.70 1.81 4.78 5.07 4.73 4.21 4.98 4.43 mmu-miR-296-3p
4.3854 5.60 6.50 5.71 7.13 8.85 8.00 7.12 7.29 6.53 mmu-miR-466i-5p
4.0231 5.41 5.87 5.23 7.23 7.67 7.69 6.26 6.55 6.35 mmu-miR-674-5p
3.7338 4.88 4.78 5.38 7.45 6.92 6.33 7.08 7.07 7.07 mmu-miR-223-3p
3.5910 5.19 5.71 5.11 6.62 7.49 7.46 5.69 5.89 5.74 mmu-miR-7033-5p
3.5821 2.91 4.73 3.39 5.41 5.51 6.12 5.19 5.41 4.73 mmu-miR-5126
3.5346 2.70 2.46 3.09 4.43 4.43 4.73 2.17 2.91 1.81 mmu-miR-30c-2-3p
3.5051 4.55 4.61 4.29 6.16 6.37 6.42 6.40 6.08 5.57 mmu-miR-3535
3.4714 7.41 7.34 6.73 8.91 9.37 8.69 10.07 9.70 9.67 mmu-miR-155-5p
3.3099 8.20 8.32 7.90 9.98 9.85 9.87 11.88 11.40 11.88 mmu-miR-15b-5p
3.2777 7.59 7.81 7.40 9.54 8.94 9.47 8.48 8.33 8.29 mmu-miR-674-3p
3.2716 3.86 3.86 3.39 5.02 5.97 5.19 3.86 4.43 4.61 mmu-miR-1195
3.2596 4.29 3.52 3.95 5.82 5.69 5.57 9.01 8.70 8.79 mmu-miR-3473e
3.2015 3.64 1.81 3.64 4.61 4.78 5.15 5.02 4.88 4.29 mmu-miR-2137
3.0506 4.49 4.55 5.38 6.26 7.02 6.12 7.46 7.60 7.56 mmu-miR-20a-3p
2.9266 4.29 4.04 4.21 5.48 5.92 5.85 7.93 7.65 7.19 mmu-miR-5121
2.9155 3.64 3.86 2.17 4.55 5.69 4.36 5.94 5.92 6.45 mmu-miR-19b-1-5p
2.9148 10.73 10.89 10.25 12.35 12.24 12.06 13.95 13.59 13.89 mmu-miR-16-5p
2.8656 6.92 7.39 7.30 8.62 8.94 8.73 8.22 8.22 8.11 mmu-miR-5099
2.8654 5.34 5.87 5.77 6.84 7.27 7.46 7.34 7.43 7.10 mmu-miR-1949
2.8614 7.27 6.83 7.12 8.67 8.87 8.31 11.09 10.79 11.03 mmu-miR-128-3p
2.8312 4.04 3.64 3.09 5.34 4.55 5.27 4.49 4.21 4.04 mmu-miR-25-5p
2.8217 7.66 7.64 7.76 9.21 9.25 9.18 9.58 9.29 9.82 mmu-miR-16-2-3p
2.7885 4.43 5.19 4.21 5.74 6.29 6.40 4.61 3.86 4.36 mmu-miR-3069-3p
2.7833 9.81 10.13 9.40 11.06 11.33 11.50 9.84 10.44 9.97 mmu-miR-690
2.4952 4.04 3.25 4.55 3.95 6.47 4.55 7.42 7.70 7.62 mmu-miR-18a-5p
2.4816 2.17 3.09 2.91 3.09 3.86 4.61 2.91 3.09 3.25 mmu-miR-504-5p
2.4037 10.77 10.27 10.77 11.90 11.98 11.86 12.05 11.77 12.19 mmu-miR-15b-3p
2.2298 7.64 8.41 7.50 8.94 9.12 9.20 7.19 6.80 6.65 mmu-miR-6516-3p
2.0972 3.09 3.52 2.91 3.52 4.55 4.55 4.49 5.11 5.15 mmu-miR-345-5p
2.0344 6.53 7.16 6.89 7.90 7.23 8.40 7.34 6.97 7.54 mmu-miR-92a-1-5p
2.0065 7.41 6.77 7.68 8.03 9.11 7.56 9.99 9.78 10.05 mmu-miR-20a-5p
2.0020 5.77 6.22 6.08 6.79 7.44 6.91 6.43 6.42 6.38 mmu-miR-149-5p
1.9867 8.41 8.32 8.65 9.22 9.99 9.11 9.76 9.65 9.37 mmu-miR-28a-3p
1.9846 11.25 11.22 11.12 12.08 12.50 12.03 11.53 11.43 11.39 mmu-miR-7a-5p
1.9736 5.02 4.36 4.36 5.15 6.06 5.51 3.75 4.13 3.75 mmu-miR-712-5p
1.9704 5.69 5.85 5.57 6.45 6.99 6.64 5.94 6.26 5.77 mmu-miR-677-3p
1.9508 5.63 5.41 6.29 6.35 7.11 6.91 7.41 7.65 8.04 mmu-miR-181c-5p
1.9500 14.66 14.52 14.63 15.66 15.55 15.57 14.84 14.58 14.54 mmu-miR-342-3p
1.9234 7.59 7.18 7.57 8.08 8.92 8.16 8.11 8.12 8.16 mmu-miR-378a-5p
1.9060 5.97 6.10 5.82 6.89 6.95 6.92 9.19 8.98 9.03 mmu-miR-3473b
1.8927 7.97 7.43 8.07 8.97 9.01 8.36 9.59 9.34 9.61 mmu-miR-194-5p
14
12
10
8
6
4
2
Log2CPM

Figure 4.2.9 (shown on preceding pages) - Top differentially expressed microRNAs between GFP⁺ and YFP⁺ γδ T cells.
(**a**) All 43 microRNAs found more expressed in GFP⁺ cells with a FDR ≤ 0.05. (**b**) Top 50 microRNAs found more expressed in YFP⁺ cells with a FDR ≤ 0.05. In both (**a**-**b**), microRNAs are ordered by decreasing fold change values (FC). FC is indicated on the left side and mature microRNA names are indicated on the right side. Values inside rectangles refer to the individual log_2CPM (Counts Per Million) values of each replicate (named A, B and C) for each sample (GFP for GFP⁺; YFP for YFP⁺ and DN for double negative GFP⁻ YFP⁻).

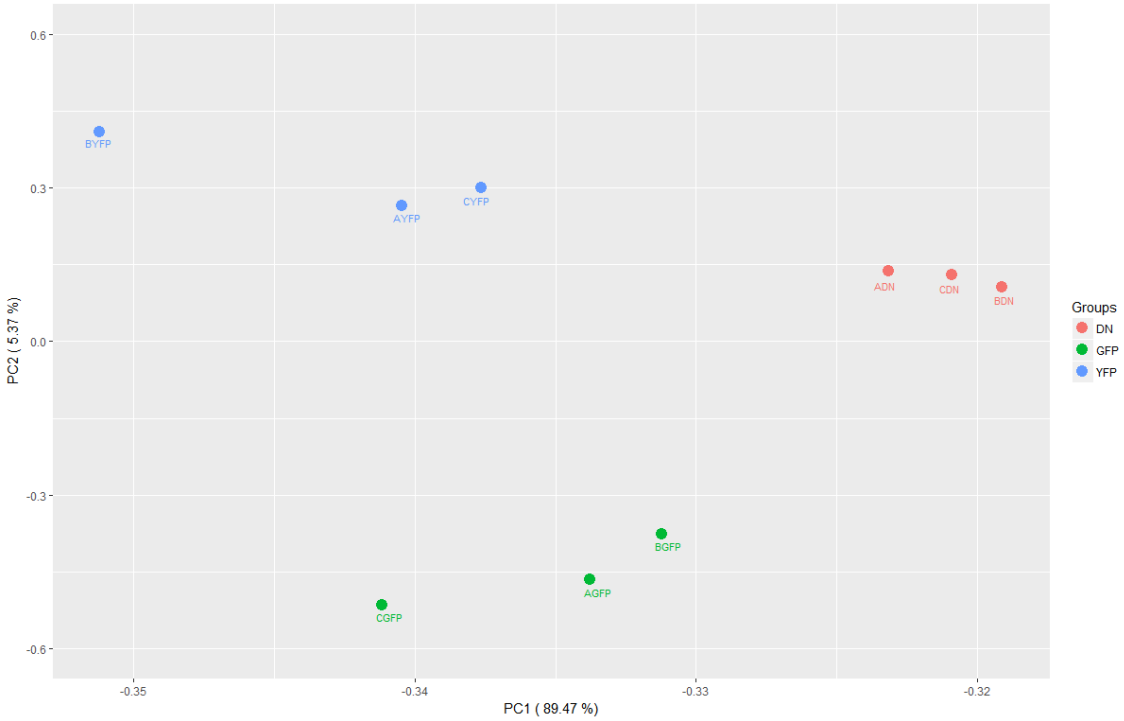


Figure 4.2.10 - Principal component analysis (PCA) plot from microRNA sequencing data analysis.
PCA of all the 9 samples used for microRNA sequencing.
Each population of cells isolated: GFP⁺ YFP⁻ (labelled GFP, in green); GFP⁻ YFP⁺ (labelled YFP, in blue) and GFP⁻ YFP⁻ (labelled DN, in red) is represented by its 3 independent samples labelled A, B and C. All 3 samples from the same cellular population cluster together and distantly from the other 2 populations.

In both groups we found several microRNAs presenting considerable fold changes and, amongst them, a small set that stood out by showing distinctively high values (FC>15). In GFP⁺ cells this set was composed by miR-183-5p (FC=45), miR-322-3p (FC=28), it's complementary strand miR-322-5p (FC=27) and miR-450b-3p (FC=25). In YFP⁺ cells the analogous microRNAs were miR-139-5p (FC=45), miR-221-3p (FC=29), miR-181a-5p (FC=22) and miR-222-3p (FC=15).

In terms of general relative abundance, inferred by the number of CPM observed, miR-150-5p was by far the most abundant microRNA in both populations although it did show a 1.8 fold enrichment in IFN-γ^+ cells. Other abundant microRNAs also enriched in IFN-γ^+ cells include miR-342-3p (FC=1.9) and miR-92a-3p (FC=1.7). In parallel, miR-24-3p (FC=1.9), miR-23a-3p (FC=2.6) and miR-146a-5p (FC=1.7) were also abundant microRNAs in both populations but showed higher expression in IL-17$^+$ cells.

4.2.2.6 - *Establishment of microRNA:mRNA networks*

In order to understand the involvement that the detected microRNAs have in the functional program of effector $\gamma\delta$ T cells we combined the data obtained from the transcriptome with the data obtained from the miRNome. First, based on the literature, we made a list of genes that we associated with either the IFN-γ or the IL-17 secreting functional subset (**Figure 4.2.11**). We then crossed this list against our transcriptomic data and we selected those that were significantly more expressed in the expected cell population and that had a fold change equal or higher than 1.5. This resulted in the generation of two lists, one with 20 genes for the IL-17 "program" and another with 9 genes for the IFN-γ "program" (**Figure 4.2.11**).

MicroRNAs reduce the amount of their target mRNAs by promoting destabilization and degradation. This is accomplished after they recognize and bind to sequence specific sites in their target's 3' UTR. Since $\gamma\delta$ T cells seemingly commit to one or the other subset, which are mutually exclusive at least under homeostatic conditions, we hypothesized that the microRNAs found in cells expressing IL-17 could inhibit the transcripts required for the IFN-γ effector phenotype, and *vice versa*. With this in mind we asked if we could find binding sites for the microRNAs with higher expression in GFP$^+$ cells in the mRNAs that are part of the IFN-γ "program" and that are more abundant in the YFP$^+$ cells. We asked the same question for the microRNAs enriched in YFP$^+$ cells and the transcripts enriched in GFP$^+$ cells. We systematize the results in two networks where we can visualize the targets of each microRNA and the interactions between them (**Figure 4.2.12**). Based on

these networks, and taking into consideration the fold change and the identity of their targets, we selected 5 microRNAs targeting the IL-17 "program" and 3 microRNAs targeting the IFN-γ "program" (**Table 4.3**). The first group, enriched in IFN-γ producing cells, is composed by miR-139-5p, miR-1949, miR-128-3p, miR-7a-5p and miR-677-3p. The second, enriched in IL-17 producing cells, comprises miR-322-5p, miR-450b-3p and miR-326-3p.

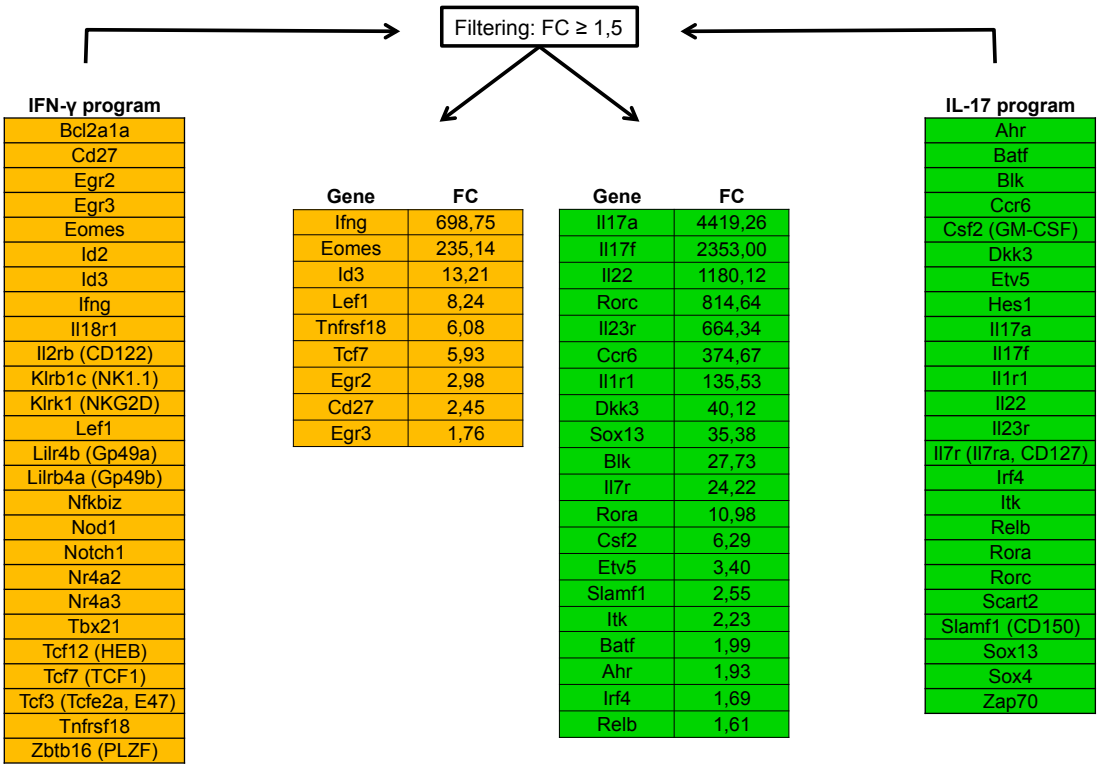


Figure 4.2.11 - List of genes belonging to the IFN-γ or IL-17 programs that are enriched in the YFP⁺ or GFP⁺ γδ T cell populations.
Tables on the far most left and right list genes that have been described to be involved in the function or identity of γδ T cells producing IFN-γ or IL-17 respectively. In the middle are the same tables but only with the genes that cleared our filtering criteria: be enriched in the correct population and have a fold change no smaller than 1.5.

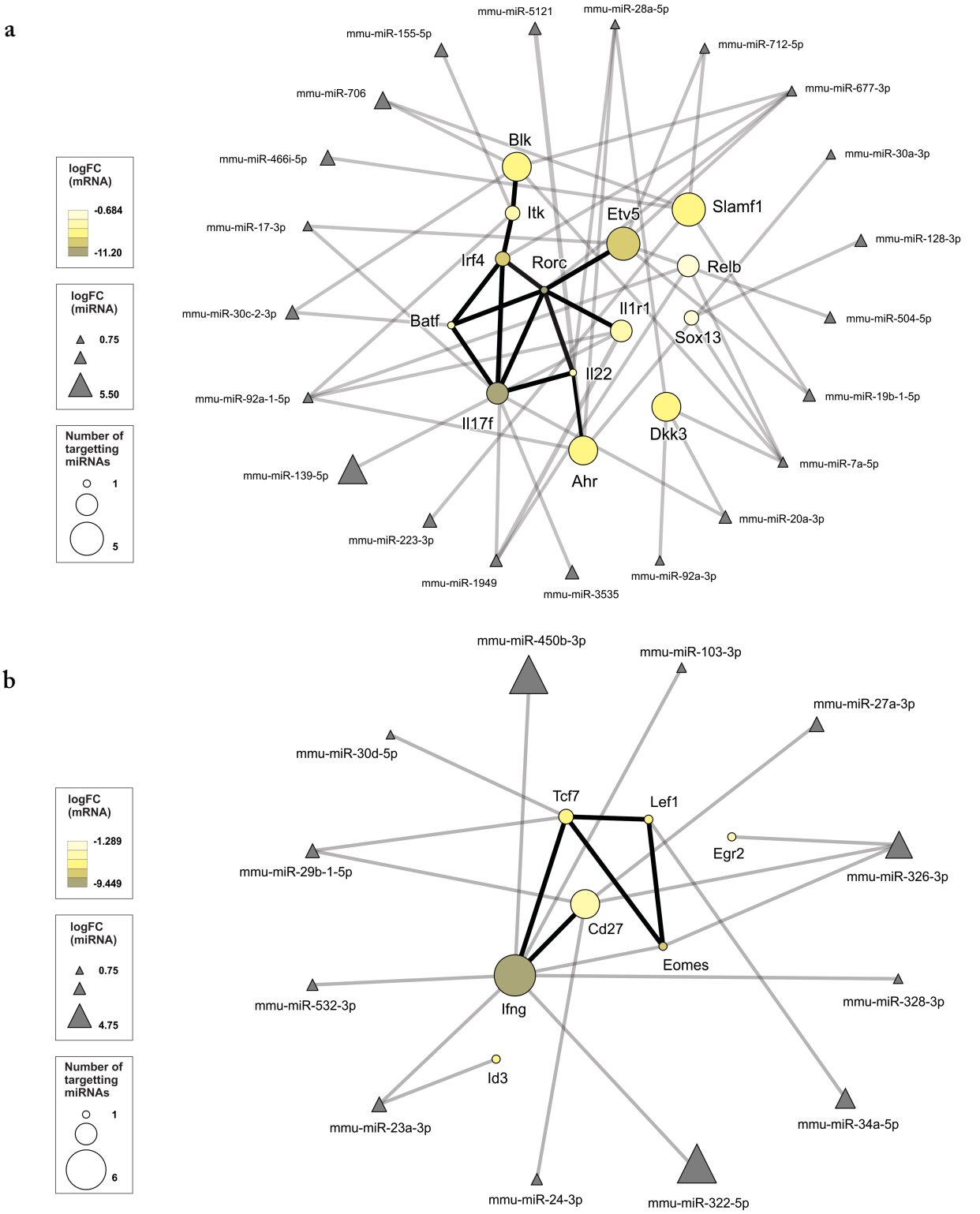


Figure 4.2.12 - Interaction networks of microRNAs and their targets.
(**a**) MicroRNAs enriched in the YFP⁺ population and their respective mRNA targets, enriched in GFP⁺ cells and described to have a role in IL-17 producing *γδ* T cell biology. (**b**) MicroRNAs with higher expression in GFP⁺ cells and their mRNA targets, enriched in YFP⁺ cells and described to have a role in IFN-*γ* producing *γδ* T cell biology. In both (**a**-**b**), grey lines denote microRNA-target mRNA interaction and dark lines denote protein-protein interaction.

Table 4.3 - List of microRNA candidates selected to be tested in functional assays.

microRNA ID	*Enriched in Population*	*Fold Change*	*Number of targets*	*Target genes*
miR-139-5p	YFP$^+$	45	1	Il1r1
miR-1949	YFP$^+$	3	3	Il1r1, Irf4, Relb
miR-128-3p	YFP$^+$	3	1	Sox13
miR-677-3p	YFP$^+$	2	4	Blk, Etv5, Irf4, Rorc
miR-7a-5p	YFP$^+$	2	4	Blk, Dkk3, Relb, Sox13
miR-322-5p	GFP$^+$	27	1	Ifng
miR-450b-3p	GFP$^+$	25	1	Ifng
miR-326-3p	GFP$^+$	7	3	Egr2, Eomes, Cd27

Note: For each candidate it is indicated the population in which they were enriched, respective fold change, number and name of the predicted targets from the microRNA:mRNA network analysis.

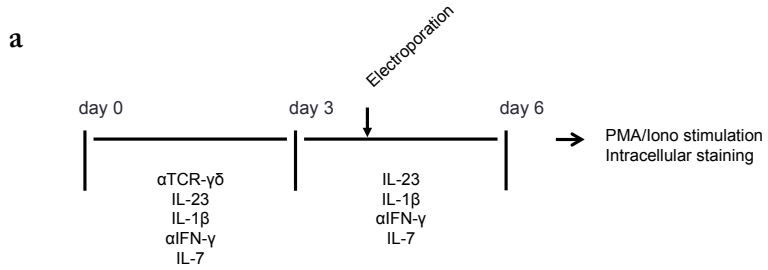
To understand which microRNAs, amongst our selected candidates, exerted a key role in the maintenance of the effector $\gamma\delta$ T cell identity or in the ability to produce their hallmark cytokines, we decided to test what the impact of altering their levels in $\gamma\delta$ T cells.

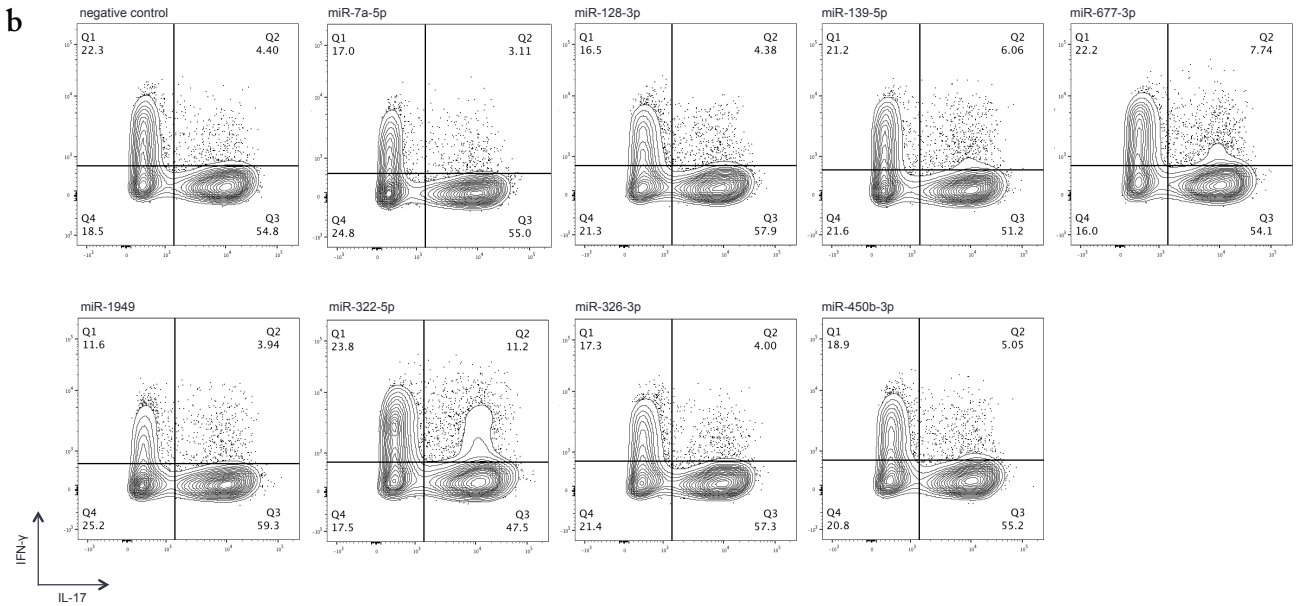
4.2.2.7 - *Impact of microRNA manipulation on cytokine expression*

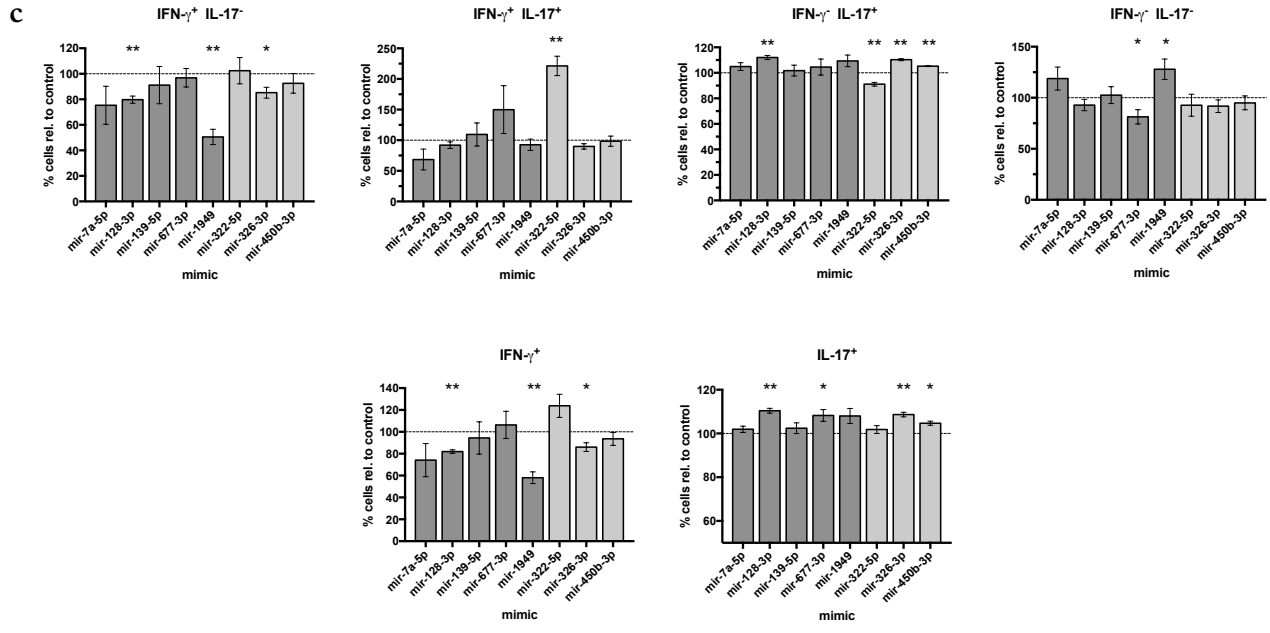
To experimentally increase or decrease the levels of our candidate microRNAs and test our hypotheses, we decided to employ the use of small molecules that are either similar to a mature microRNA and thus mimic their action (called mimics) or possess a partially complementary sequence that will stably bind and sequester the mature microRNAs impeding their ability to bind to their targets transcripts and exert their function (called inhibitors). To do so we followed the workflow outlined in **Figure 4.2.13 a**. We isolated cells from peripheral lymph nodes and spleen of C57BL/6 mice and expanded them *in vitro* for 3 days in the presence of α*(anti)*TCR-$\gamma\delta$, IL-23, IL-1β, αIFN-γ and IL-7. After 3 days we reseeded the cells using a similar cocktail but without αTCR-$\gamma\delta$. At day 4 cells were transfected with the mimics or inhibitor using electroporation and 2 days later their ability to produce IFN-γ and IL-17 was addressed by intracellular staining followed by flow cytometry. The ability or not of the microRNA candidates to post-transcriptionally

control cytokine expression was revealed by the experiments in which we increased the intracellular concentration of the microRNA by introducing mimic molecules into the cells (**Figure 4.2.13 b and Figure 4.2.13 c**) or where we inhibited their action by introducing inhibitor molecules specific for each microRNA (**Figure 4.2.13 d and Figure 4.2.13 e**). Modulation impacted mainly on the ability of cells to produce IFN-γ. MiR-326-3p, miR-7a-5p and miR-1949 were able to reduce IFN-γ expression, miR-128-3p inhibited IFN-γ and promoted, slightly, IL-17 expression; and finally miR-322-5p promoted $\gamma\delta$ T cell plasticity by allowing an increase in IL-17$^+$ cells that co-expressed IFN-γ. The changes observed for each candidate are described and discussed further in the discussion section.

Alternatively, we transduced the cells with viral particles containing a vector with a GFP reporter overexpressing each microRNA. The protocol, outlined in **Figure 4.2.14 a**, is similar to the previous one with the exception that cells are transduced at day 4 and day 5; from day 6 onwards cells are incubated with medium supplemented with only IL-7 and αIFN-γ and restimulation and intracellular staining is performed at day 9. These results, although preliminary (as shown by the absence of statistical analysis) were able to corroborate the results obtained with the mimics, namely the ability of miR-128, miR-7 and miR-326 to reduce IFN-γ expression, and the ability of miR-322 to induce it in cells co-expressing IL-17 (**Figure 4.2.14 b and c**).


a
Electroporation
day 0
day 3
day 6
PMA/Iono stimulation
Intracellular staining
αTCR-γδ
IL-23
IL-1β
αIFN-γ
IL-7
IL-23
IL-1β
αIFN-γ
IL-7


b
negative control
miR-7a-5p
miR-128-3p
miR-139-5p
miR-677-3p
miR-1949
miR-322-5p
miR-326-3p
miR-450b-3p
IFN-γ
IL-17


c
IFN-γ+ IL-17-
IFN-γ+ IL-17+
IFN-γ- IL-17+
IFN-γ- IL-17-
IFN-γ+
IL-17+
% cells rel. to control
mimic
mir-7a-5p
mir-128-3p
mir-139-5p
mir-677-3p
mir-1949
mir-322-5p
mir-326-3p
mir-450b-3p

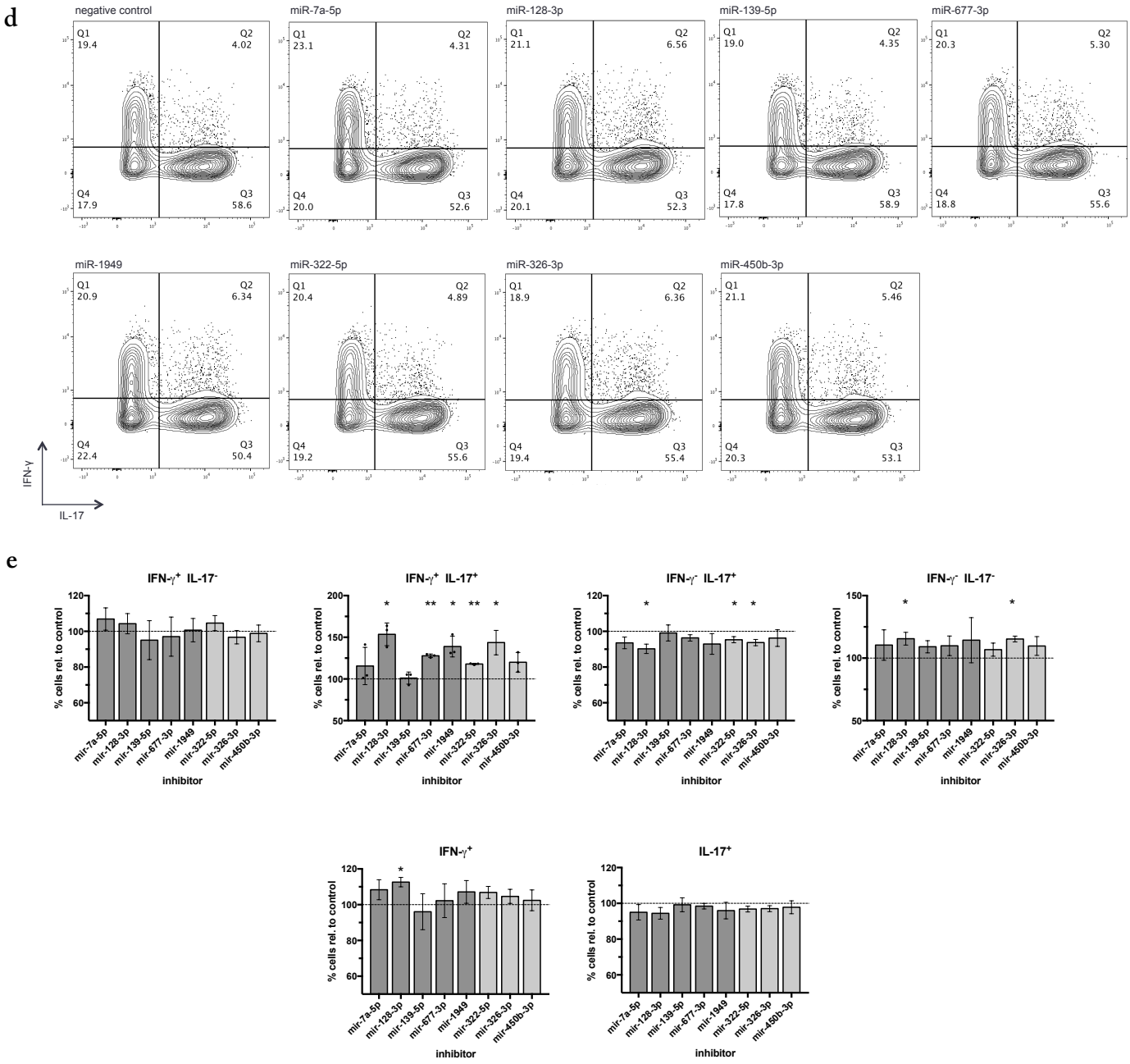


Figure 4.2.13 - Altering microRNAs levels impacts on cytokine production by *γδ* T cells.
(**a**) Workflow of the strategy used. (**b**) Examples of flow cytometry plots showing IFN-*γ* *vs* IL-17 expression by *γδ* T cells transfected with the indicated microRNA mimic. (**c**) Frequency of cytokine expressing populations upon transfection with the indicated microRNA mimic. (**d**) Examples of flow cytometry plots showing IFN-*γ* *vs* IL-17 expression by *γδ* T cells transfected with the indicated microRNA inhibitor. (**e**) Frequency of cytokine expressing populations upon transfection with the indicated microRNA inhibitor. For an easy reference, bars in (**c**) and (**e**) are depicted in dark grey for microRNA candidates predicted to target the IL-17 program, and depicted in light grey for microRNA candidates predicted to target the IFN-*γ* program. Results in (**c**) and (**e**) are normalized to the respective mimic or inhibitor negative control. (n=3)

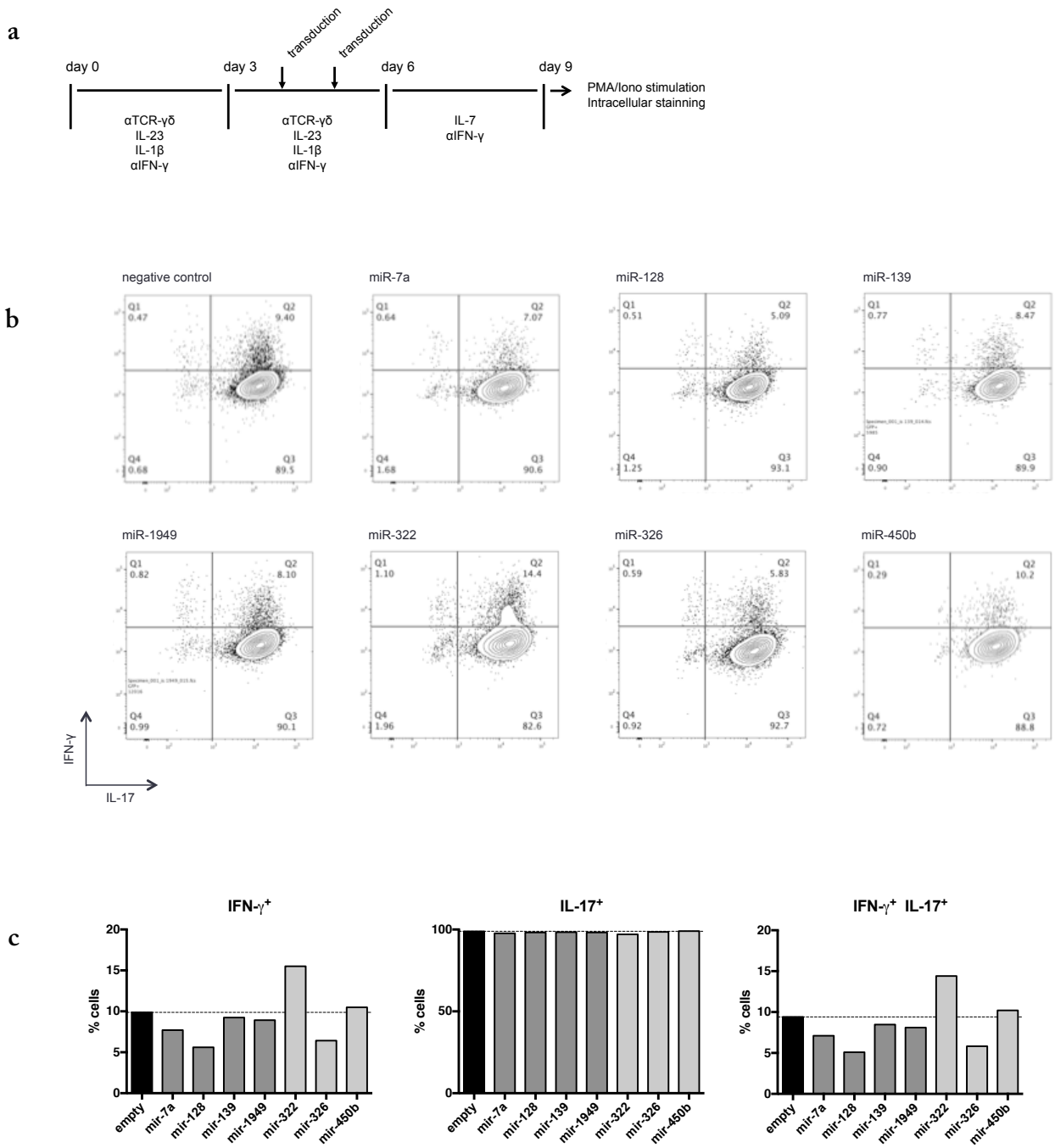


Figure 4.2.14 - Overexpression of microRNAs impacts on *γδ* T cell ability to produce IFN-*γ*.
(**a**) Workflow of the strategy used. (**b**) Examples of flow cytometry plots showing IFN-*γ* *vs* IL-17 expression by *γδ* T cells overexpressing the indicated microRNA precursor. (**c**) Frequency of cytokine expressing populations upon transduction. For an easy reference, bars in (**c**) are depicted in dark grey for microRNA candidates predicted to target the IL-17 program and depicted in light grey for microRNA candidates predicted to target the IFN-*γ* program and in black for the control empty vector. (n=1).

2.2.3 - Discussion

MicroRNAs have been widely reported to play important roles in the biology of many cell types, including those that are part of the immune system. In the T cell field, most research on microRNAs has been conducted on $CD4^+$ $\alpha\beta$ T cells and revealed the impact of microRNA-mediated control of gene expression in a variety of processes such as activation, proliferation, survival, differentiation and control of effector functions, among others. So far there have been very few studies that investigated the role of microRNAs in the biology of $\gamma\delta$ T cells. Taking into consideration the importance microRNAs have in their $\alpha\beta$ counterparts, and virtually every cell in the organism, we decided to study microRNAs in effector $\gamma\delta$ T cells.

Since, as mentioned before, few studies have been done on this topic, and in order to assess the microRNA landscape in the two main effector subsets of $\gamma\delta$ T cells, IL-17 producers and IFN-γ producers, we started by characterizing their miRNome using next generation sequencing. We found 103 microRNAs to be differentially expressed between the two populations. IL-17 producing cells showed 43 microRNAs with significantly higher expression and 60 microRNAs with significant lower expression compared to IFN-γ producing $\gamma\delta$ T cells.

In a recent report by our group, miR-146a-5p and miR-146b-5p were described to be enriched in $CD27^-$ $CCR6^+$ $\gamma\delta$ T cells, a phenotype that associates with the potential to produce IL-17 [217]. In accordance with this observation, we found both these microRNAs to be more expressed in the GFP^+ population (FC=1.7 and FC=1.5 respectively), while their 3p complementary strands have extremely low or undetectable expression. However, these two mature microRNA species were the only ones found to be enriched in the $CD27^-$ $CCR6^+$ population when compared to the $CD27^+$ population, which had 33 enriched microRNAs; making a total of 35 microRNAs found differentially expressed in this study that used a limited microarray platform [217]. By contrast, in the current work based on NGS, we were able to identify 103 differentially expressed microRNAs, with 43 enriched in cells with the $IL\text{-}17^+$ phenotype. NGS shows a dynamic range with a magnitude far greater than that of microarrays and allows the detection of low-abundant

microRNAs that would, most likely, be lost in the noise inherent to microarrays. Our strategy also does not rely on the binding of sequence specific probes, which can introduce some bias in the detection of the microRNA and in the signal amplification. On the other hand, by relying on cytokine reporters instead of extracellular markers to isolate the effector cell populations of interest, we were able to achieve a much higher degree of purity, which may have revealed enrichment in genes on cells expressing the cytokine that would otherwise be unnoticeable if they were mixed with others that did not show the same enrichment or that had the opposite trend for that same gene.

Amongst the first reports on microRNAs in γδ T cells is a study that described the expression of miR-133b and miR-206 as transcriptionally co-regulated along with the closely located *Il17a/f locus* [213]. The authors used several markers such as CCR6, CD27 and a RORγt reporter to isolate the cells where they probed the expression of these microRNAs. Even though our transcriptomic data showed *Ccr6* and *Rorc* expression exclusively in GFP$^+$ cells, and these same cells present the lowest *Cd27* expression of the three populations analysed, we unexpectedly were not able to find neither miR-133b nor miR-206 species in our differential expression analysis. However, we could find very low numbers of raw counts, essentially only for miR-206-3p and only on GFP$^+$ cells, in accordance with the observations in this paper. The low or inexistent counts (**Table 4.4**) justify why we did not find these microRNAs in our differentially expression analysis; as well as their lack of any detectable role in effector γδ T cells, as acknowledged in that previous report [213].

Another microRNA that has been studied in γδ T cells is miR-181a/b-1. MiR-181 has been described to be involved in promoting TCR signalling, namely by reducing the TCR signalling threshold. Although the absence of this microRNA does not alter the development or differentiation of γδ T cells, it is nonetheless interesting to find it enriched in YFP$^+$ cells (miR-181a-5p FC=22), since an increased TCR signal strength has been associated with the differentiation of IFN-γ secreting γδ T cells [222].

Table 4.4 - Expression of miR-133b and miR-206 in sorted γδ T cells.

Mature miRNA	*AGFP*	*BGFP*	*CGFP*	*AYFP*	*BYFP*	*CYFP*	*ADN*	*BDN*	*CDN*
mmu-miR-133a-5p	0	0	0	0	0	0	0	0	0
mmu-miR-133a-3p	0	0	0.50	0	0	0	0	0	0
mmu-miR-206-5p	0	0	0	0	0	0	0	0	0
mmu-miR-206-3p	3.0	10.0	5.0	0	0	0	0	1.0	0
mmu-miR-146a-5p	20439.5	16251.5	22889.5	5051.5	4392.5	8135.0	5682.0	5910.5	10370.5
mmu-miR-423-3p	837.0	666.0	880.0	405.0	394.0	520.0	909.0	859.0	1192.0

Note: We did not detect miR-133b or miR-206 in our expression data. This was due to the very low expression levels of these two microRNAs. Mir-423-3p, non differentially expressed but easily detected, is commonly used as a housekeeping gene for microRNA RT-qPCR and is shown here to provide some reference. MiR-146a-5p, mentioned before as being enriched in GFP$^+$ cells, is also shown as a reference.

With the information of which microRNAs were enriched in IL-17$^+$ and IFN-γ^+ $\gamma\delta$ T cells we then aimed at understanding which among them exerted a key role in the maintenance of effector cell identity and the ability to produce hallmark cytokines. After selecting some microRNA candidates, based on their expression profile and predicted targets, we tested the impact of changing their levels on the ability of $\gamma\delta$ T cells to produce the cytokines that define the two effector subsets.

The microRNAs miR-322-5p, miR-326-3p and miR-450b-3p were selected as they were enriched in GFP$^+$ cells and had among their predicted targets genes described to be involved in the IFN-γ program of $\gamma\delta$ T cells. One could thus expect that increasing their levels, by introducing mimics into the cell or by overexpressing its precursor form, would lead to a reduction in the ability to produce IFN-γ. This was indeed the case observed for miR-326-3p (FC=7, target=3) whose gain-of-function led to a significant reduction in the frequency of IFN-γ^+ cells (~15%), especially in the IFN-γ^+ IL17$^-$ population, in both mimic or overexpression experiments (**Figure 4.2.13** and **Figure 4.2.14**). Consistently, upon using miR-326-3p inhibitors we observed an increase in the production of IFN-γ, but only in cells that also express IL-17 (**Figure 4.2.13**).

The results obtained with miR-326-3p fit a simple model where increasing the levels of a microRNA that targets IFN-γ expression leads to decreased percentages of cells expressing IFN-γ. This effect should be bigger on cells that belong to the IFN-γ effector subset since

cells belonging to the IL-17 subset have higher amounts of the microRNA, and increasing the levels of a microRNA that is already abundant will probably generate milder results than if the microRNA is scarce. Following the inverse logic, when reducing the levels of this microRNA we expect an increase in the amount of cells producing IFN-γ; and this should be more noticeable in the cells where this microRNA is more expressed rather than in those that already have reduced expression. This is exactly what we observed for miR-326-3p in all the assays performed.

However this was not the case for the other microRNAs in this group. Altering miR-450b (FC=25, target=1) levels did not cause any effect in the ability of the cells to produce the two cytokines tested, neither when its levels were increased, nor when they were decreased (**Figure 4.2.13**). MiR-322-5p (FC=27, target=1) showed a very different and unexpected result. There was a striking increase (~121%) in the fraction of effector IL-17$^+$ cells that co-produced IFN-γ, commonly referred to as double producers, when we increased miR-322-5p intracellular levels by mimic electroporation (**Figure 4.2.13**). Such an increase was also observed in the viral-mediated overexpression of this microRNA (**Figure 4.2.14**). Puzzlingly, a small increase was also observed when using an inhibitor of miR-322-5p (**Figure 4.2.13**), but this increase was much smaller (~18%). Although this microRNA has a predicted binding site in the IFN-γ transcript, these results suggest that in γδ T cells, miR-322-5p exerts its role through several other targets and that its cellular levels may be strictly regulated. In fact, this microRNA might be acting in a different way than the one we based our approach on. By targeting genes responsible for the repression of IFN-γ, miR-322-5p may ensure that the IFN-γ program is not completely shut off, allowing the IL-17 effector subset to be plastic and co-produce IFN-γ under certain (namely inflammatory) conditions. However we still need to identify which are the mRNA targets responsible for this phenotype; and it would be interesting to perform a similar experiment under conditions that induce a higher percentage of double producers.

The microRNAs miR-7a-5p, miR-128-3p, miR-139-5p, miR-677-3p and miR-450b-3p were selected because they were enriched in YFP$^+$ cells and had amongst their predicted targets genes described to be involved in the IL-17 program on γδ T cells. The microRNA with the biggest fold change in this group was miR-139-5p (FC=45, target=1), which was very abundant in YFP$^+$ cells. Disappointingly, changes in its levels did not result in any

alteration in the ability of γδ T cells to produce IFN-γ or IL-17, thus behaving like the negative controls of the experiments (**Figure 4.2.13**). MiR-677-3p also did not show any noteworthy and consistent effects (**Figure 4.2.13**). Given their high expression in IFN-γ$^+$ cells (and high differential expression) these microRNAs may play important roles in γδ T cells but in cellular processes different from the ones assessed here.

We also described a set microRNAs that, contrary to the expectation of our approach, function as regulators of IFN-γ expression in IFN-γ$^+$ cells (even despite having predicted binding site in genes associated with the IL-17 program). Although the amplitude of impact or statistical significance is not the same for all the microRNAs in this set, they do tend to cause similar effects. MiR-1949, miR-128-3p and miR-7a-5p showed an ability to reduce IFN-γ expression in gain-of-function assays, and conversely increased IFN-γ expression in loss-of-function experiments (**Figure 4.2.13**). MiR-1949 presented the strongest impact (~50%) compared to the other two microRNAs (20%) (**Figure 4.2.13**). MiR-128-3p is particularly interesting, since it simultaneously reduced IFN-γ (~20%) and enhanced IL-17 slightly (~10%), which implicated it in a complex network that controls γδ T cell effector functions (**Figure 4.2.13**). Since the effects of different microRNAs can add up it would be interesting in the future to simultaneously modulate the expression of this set of microRNAs to see if a synergetic effect is observed that leads to an even greater change in the ability to produce IFN-γ.

The fact that these microRNAs were able to reduce IFN-γ and were found more enriched in YFP$^+$ cells, indicates they are part of a post-transcriptional control system operating in IFN-γ producing γδ T cells. Although the relevant mRNA targets behind these phenomena still need to be identified, some of these microRNAs have interesting predicted targets among those we selected as part of the "IFN-γ program". We can find predicted binding sites for miR-128-3p in the 3' UTRs of genes like *Id2* or *Ifng,* and also in the coding sequence (CDS) of *Tbx21*. The 3' UTR is the region commonly accepted to be where microRNAs bind in the majority of the cases. However it is not impossible, and there are some examples in the literature, for the microRNAs to exert their function by binding to either the CDS or 5' UTR of a transcript, and so, several prediction tools now also include these regions. Using microT-CDS to visualize the microRNA:mRNA

interactions, we find that amongst the two predicted binding site within the 3' UTR of *Ifng*, one of them shares homology with the human genome. For miR-7a-5p, some algorithms indicated potential binding sites in *Eomes*. For miR-1949 the 3' UTR of *Ifng* itself, together with the 3' UTR of *Lef1*, *Egr2*, and *Eomes* were all predicted to have binding sites for this microRNA, but only by the miRWalk algorithm, so they might be false positives. Even so it is still an interesting observation that could be tested using a luciferase assay for example. As for miR-326-3p, that also showed the ability to reduce IFN-γ expression but is more expressed in the IL-17 producers, there are some very interesting targets predicted by several algorithms that could directly explain the IFN-γ reduction observed, namely *Tbx21* and *Eomes*, making it one of the more promising candidates to follow up in future studies.

Overall, the more interesting differences described before relate to changes in expression of IFN-γ. The fact that IL-17 expression was not easily changed by microRNAs when compared to IFN-γ might be due to a higher expression level of its mRNA; or a peculiarity of the *in vitro* protocol used, which promotes a strong and stable IL-17 effector phenotype in $\gamma\delta$ T cells that may resist changes in a single microRNA. In fact, this protocol is a modification of one recently published designed specifically to expand the IL-17-producing $\gamma\delta$ T cell subset, which were previously very difficult to maintain *in vitro* [223]. The original protocol was actually used in our virus overexpression experiment (**Figures 4.2.14 a**). As can be appreciated in the flow cytometry plots, this protocol is very good for its purpose, since virtually all cells are expressing IL-17. We reckoned this would not allow us to test IFN-γ production or to see increases in IL-17 expression levels, and thus invested some time and effort to moderate the IL-17 polarization, ending up with the protocol depicted in **Figure 4.2.13 a**. Due to this technical challenge, the viral mediated overexpression experiment, which was only performed once, is currently being repeated with the six days protocol from **Figure 4.2.13 a**, transducing the cells at day 4 and analysing their cytokine expression at day 6. We believe this will provide us with valuable information to be combined with the data described here.

In sum, our data demonstrated that some of the selected microRNAs are part of the mechanisms that control the functional ability of $\gamma\delta$ T cells to produce cytokines. Of

particular interest are miR-326-3p, miR-7a-5p and miR-1949, with their ability to limit IFN-γ expression; miR-128-3p that inhibits IFN-γ and promotes IL-17; and miR-322-5p that promotes functional plasticity by promoting IL-17$^+$ cells co-expressing IFN-γ. These are the candidates on which we will be focusing our future investigation, including the modulation of their expression levels *in vivo*.

Besides addressing the roles of microRNAs on effector $\gamma\delta$ T cell differentiation, we also sequenced the transcriptome of our subsets of interest. It is accepted that the identity of a given cell type is largely determined by its transcriptome, and this is the actual target of the microRNA machinery. Our transcriptomic analysis showed 7882 genes with differential expression levels between the two subsets. Besides looking at genes already described in the $\gamma\delta$ T cell literature (and which could be targets of our candidate microRNAs), we also used our transcriptome data to generate a more global picture, namely revealing particular pathways and processes differentiating the two effectors subsets, on the basis of GO Slim and Pathway Enrichment Analysis.

The analysis revealed several differences between the two effector populations but the most striking one was perhaps the potential of the cells to sense and interact with their environment. IL-17-producing $\gamma\delta$ T cells showed enormous potential for environmental information processing, as illustrated in **Table 4.1** by the 17 pathways enriched in GFP$^+$ cells that are involved in signal transduction or signalling molecules and interaction. They presented enrichment for many membrane receptors of various types, and in parallel also showed an enrichment for a large number of diverse signalling pathways, which collectively should allow them to detect and integrate multiple signals and respond accordingly. Many of these signalling pathways and receptors are, not surprisingly, related with immune known functions. **Figure 4.2.15** was based on the cytokine-cytokine receptor interaction KEGG pathway (**Figure 4.2.16)** and is intended to provide a summary of the cytokine receptors and respective ligands found enriched in IL-17$^+$ $\gamma\delta$ T cells. These signals need to be integrated into signalling networks and **Figure 4.2.17** shows the KEGG chemokine signalling pathway with the genes found more expressed in the IL-17$^+$ cells labelled in red text. In **Table 4.5** and **Table 4.7** we can find several immune related pathways such as interleukin signalling (like IL-17, IL-2, IL-9, IL-15, amongst others), interferon

signalling, chemokine signalling, Toll-like receptor signalling or NOD-like receptor signalling pathways, amongst others, whose elements are significantly enriched and represented in IL-17 producing $\gamma\delta$ T cells.

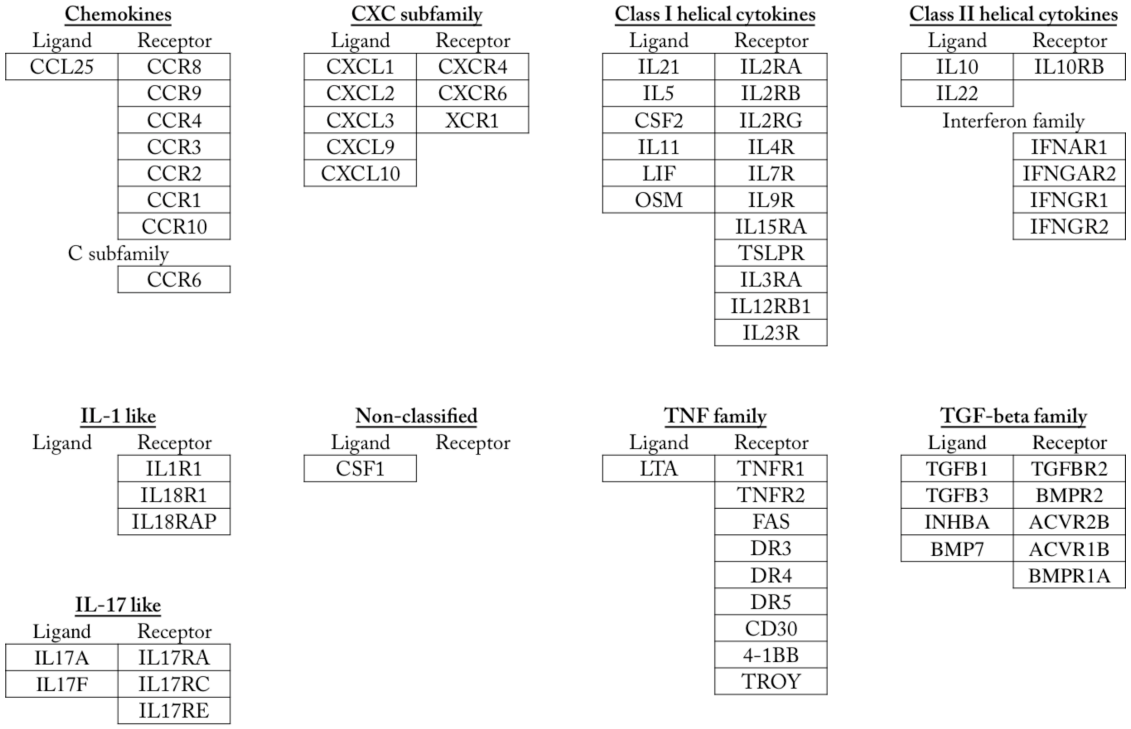


Figure 4.2.15 - Cytokine-cytokine receptor interaction for genes enriched in GFP$^+$ $\gamma\delta$ T cells T cells.
Genes indicated in the figure above showed significantly higher expression in the IL-17 secreting subset and are grouped by family. Figure is based on the KEGG pathway analysis, more specifically on the cytokine-cytokine receptor interaction pathway.

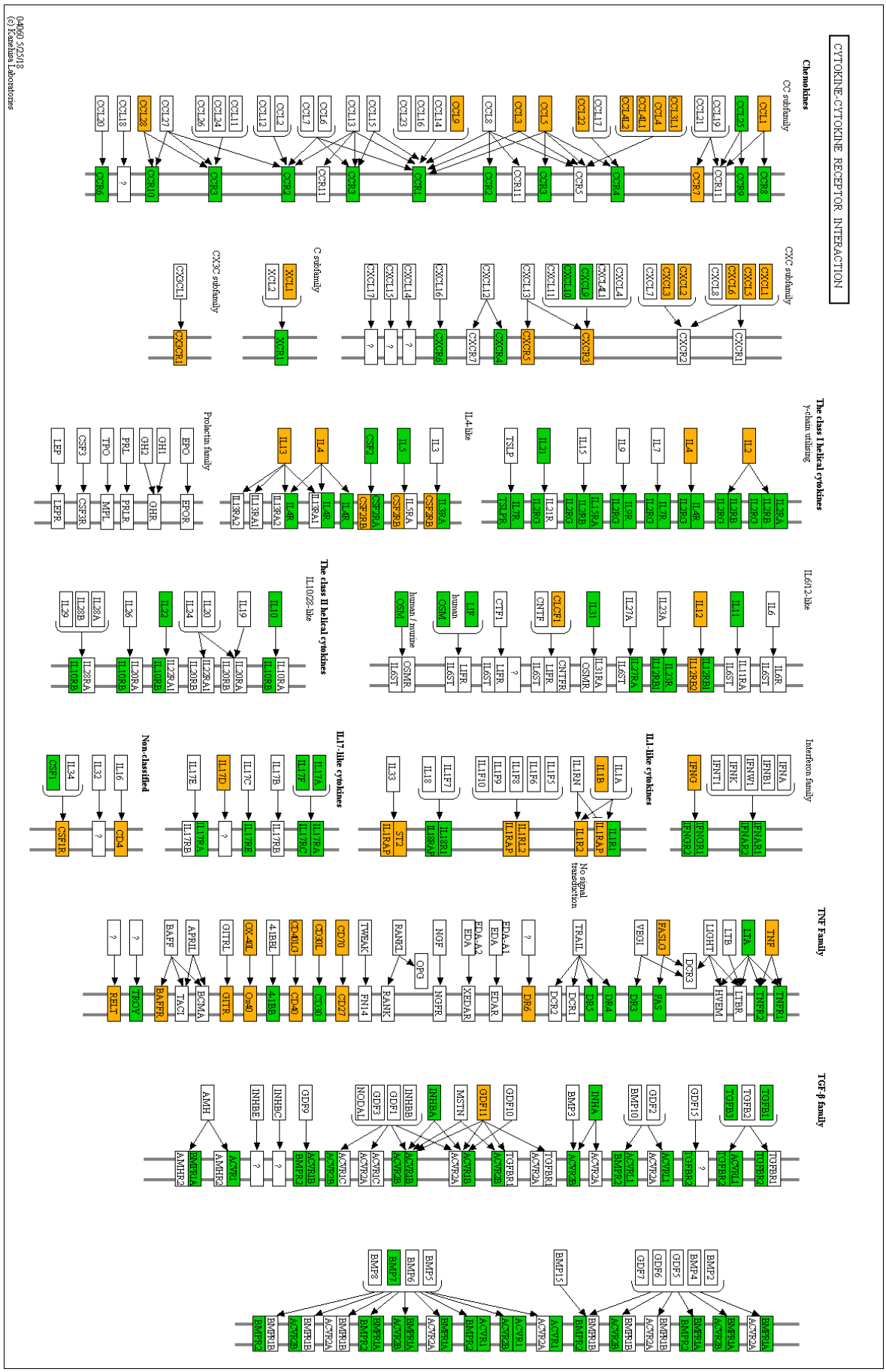

CYTOKINE-CYTOKINE RECEPTOR INTERACTION
04060 5/25/18
(c) Kanehisa Laboratories
Chemokines
CC subfamily
CXC subfamily
C subfamily
CX3C subfamily
The class I helical cytokines
γ-chain utilising
IL4-like
Prolactin family
IL6/12-like
The class II helical cytokines
IL10/28-like
Interferon family
IL1-like cytokines
No signal transduction
TNF Family
TGF-β family
IL17-like cytokines
Non-classified

Figure 4.2.16 (shown on preceding page) - Cytokine-cytokine receptor interaction (KEGG) for genes enriched in GFP⁺ or YFP⁺ γδ T cells.
KEGG cytokine-cytokine receptor interaction pathway with genes found more expressed in the IL-17⁺ cells labelled with a green background and genes found more expressed in the IFN-γ⁺ cells labelled with an orange background.

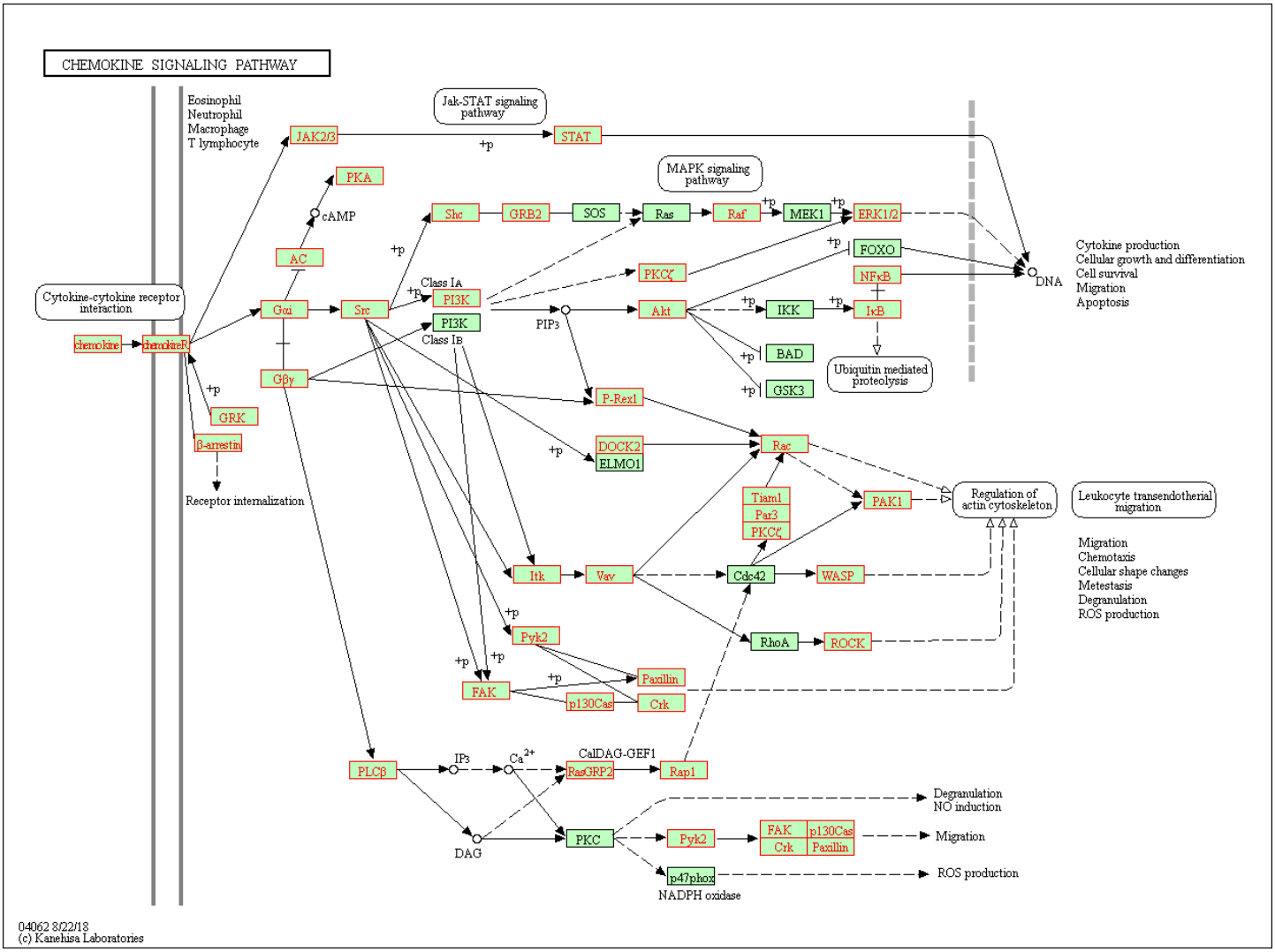
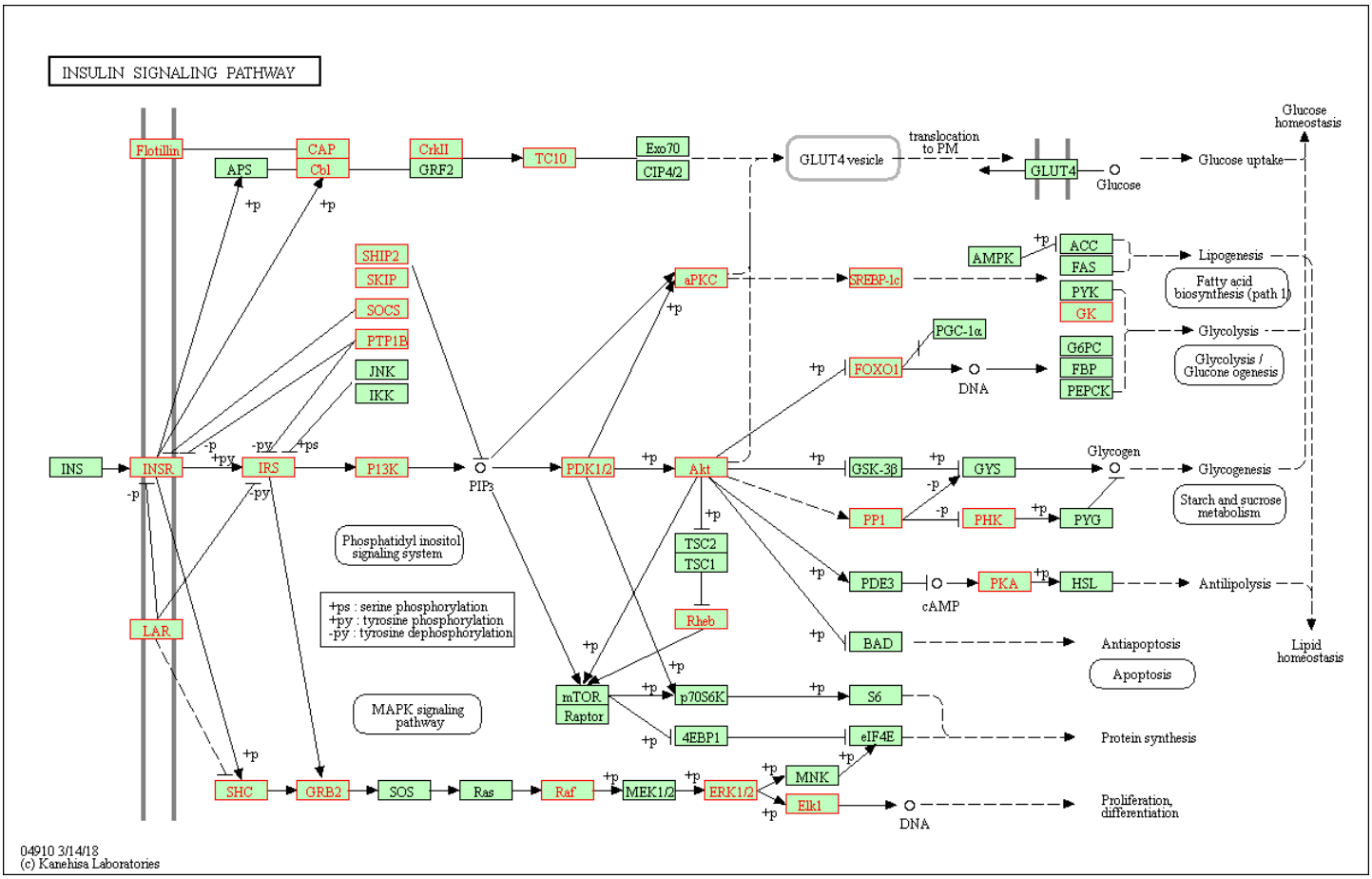


Figure 4.2.17 - Cytokine signalling pathway (KEGG) for genes enriched in GFP⁺ γδ T cells.
Graphic representation of the position and interactions of the genes involved in the KEGG chemokine signalling pathway with genes found more expressed in the IL-17⁺ cells labelled in red text.

Interestingly, our data also suggests that, among γδ T cells, IL-17 producers may be uniquely capable of integrating stimuli of non-immune nature, since they were enriched for hormone receptors (e.g. *Calcrl*), prostanoid receptors (e.g. *Ctger4*, *Ptgfrn* and *Ptgir*),

leukotriene receptors (*Ltb4r1* and *Cycltr2*); amino acid receptors (e.g. *Gabbr1* and *Gabbr2*) and even vitamin and oxysterol receptors (e.g. *Rarg*, *Vdr*, *Gp183*). In fact, several signalling pathways within the endocrine system were found to be enriched, namely insulin (**Figure 4.2.18**), relaxin, GnRH (Gonadotropin-Releasing Hormone), thyroid hormone, estrogen and oxytocin signalling pathways (**Table 4.5** and **Table 4.7**). From the nervous system many elements of the glutamatergic (**Figure 4.2.19**), cholinergic and GABAergic synapses were found to be enriched in IL-17 producers (**Table 4.5** and **Table 4.7**).

Figure 4.2.18 - Insulin signalling pathway (KEGG) for genes enriched in GFP$^+$ $\gamma\delta$ T cells.
Graphic representation of the position and interactions of the genes involved in the KEGG insulin signalling pathway with genes found more expressed in the IL-17$^+$ cells labelled in red text.



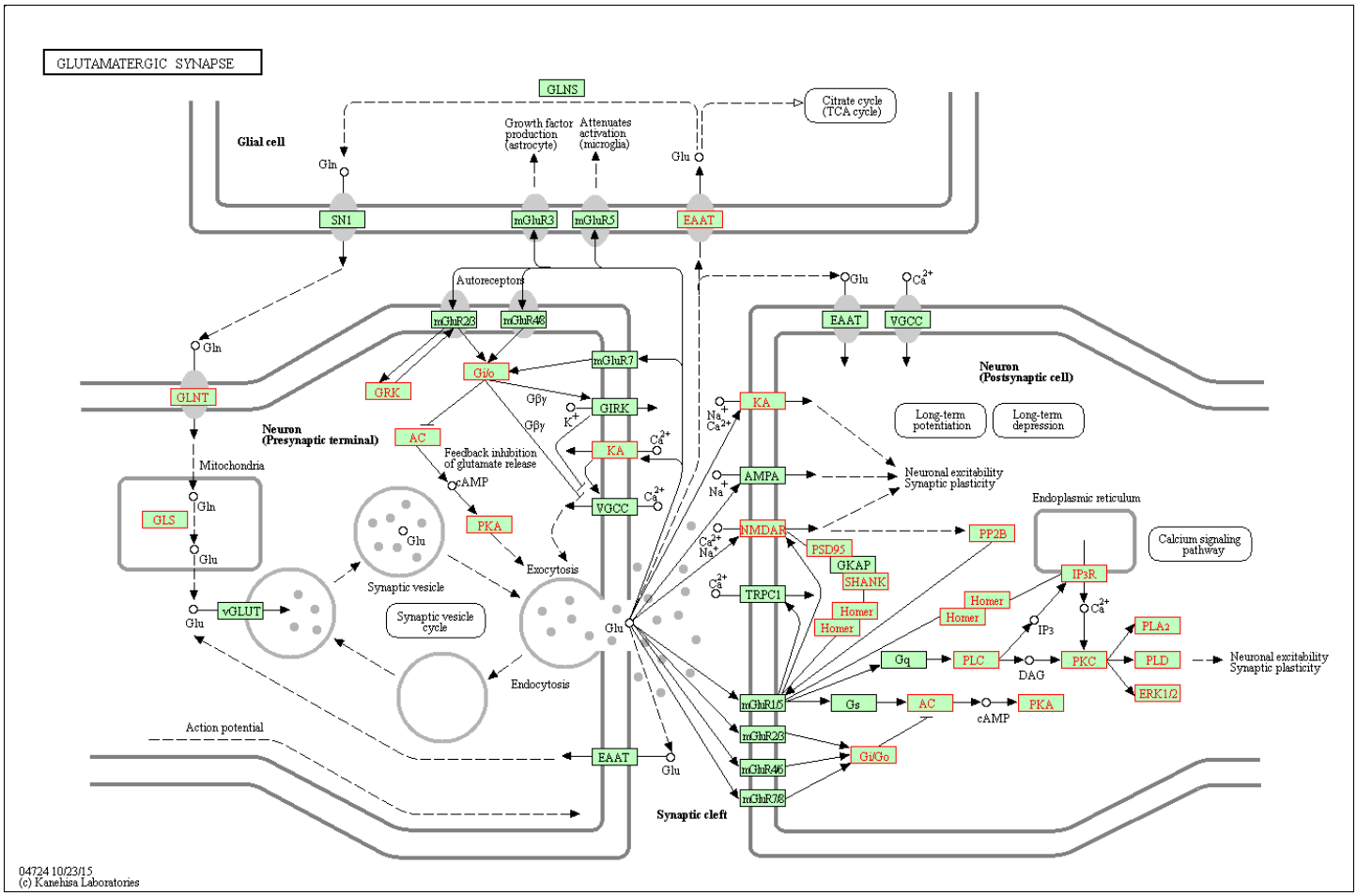


Figure 4.2.19 - Glutamatergic synapse (KEGG) for genes enriched in GFP⁺ γδ T cells.
Graphic representation of the position and interactions of the genes involved in glutamatergic synapse, with genes found more expressed in the IL-17⁺ cells labelled in red text.

Our findings that IL-17⁺ γδ T cells have receptors for molecules used within the immune, nervous or endocrine system in addition to receptors for nutrient-derived metabolites and lipid molecules, raises the possibility that these cells may be able to communicate with other systems within the body, which would likely allow them to participate in the homeostasis of such systems. In fact, this new knowledge can be used to raise various new questions. For example can IL-17 producing γδ T cells detect signals employed by different systems and of such diverse nature? How do they respond to each one? Do they leave the thymus with this ability or do they develop it in the periphery? Is it different between cells located in different tissues? In my opinion all of these are extremely interesting questions to be tackled.

Another aspect that appears to be unique to IL-17 producers is the metabolism of lipids and their use as signalling molecules. We detected several enriched pathways, namely for phosphatidylinositol signalling (**Figure 4.2.20**), shingolipd signalling pathway and phospholipase D signalling pathway. In parallel we find that pathways for the metabolism of inositol phosphate, metabolism of sphingolipids and steroid biosynthesis are also enriched in these cells (**Table 4.5** and **Table 4.7**).

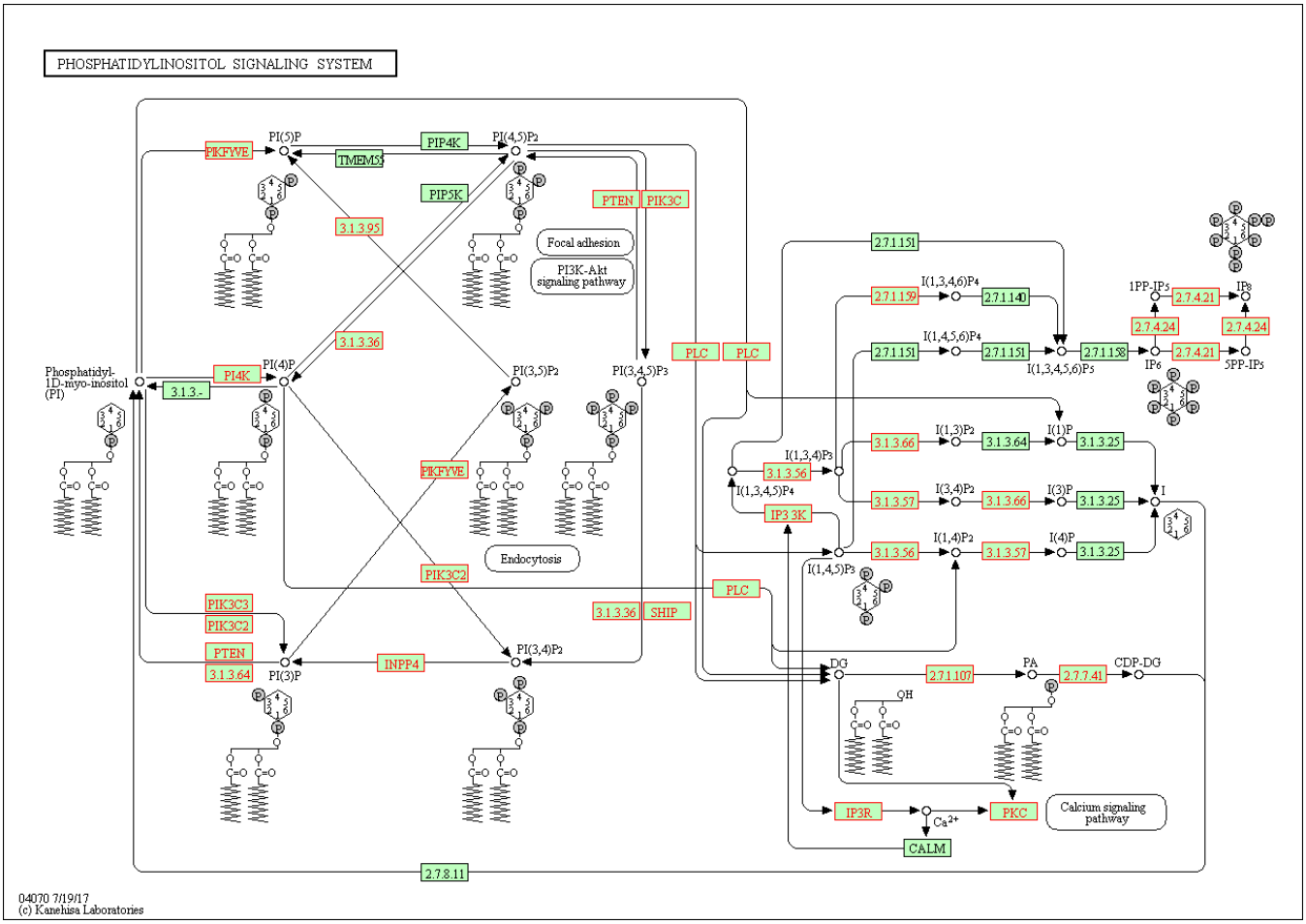


Figure 4.2.20 - Phosphatidylinositol signalling system (KEGG) for genes enriched in GFP⁺ γδ T cells.
Graphic representation of the position and interactions of the genes involved in the phosphatidylinositol signalling system with genes found more expressed in the IL-17⁺ cells labelled in red text.

This propensity for lipid metabolism may be related with the fact that these cells seem to be very rich in intracellular vesicles and trafficking, which requires the production of lipid

membranes. This as inferred by data obtained from the GO Slim analysis but also from the Pathway Enrichment Analysis where we find terms such as endocytosis, phagosome, lysosome, vesicle-mediated transport including traffic between the golgi and the ER (**Table 4.5** and **Table 4.7**).

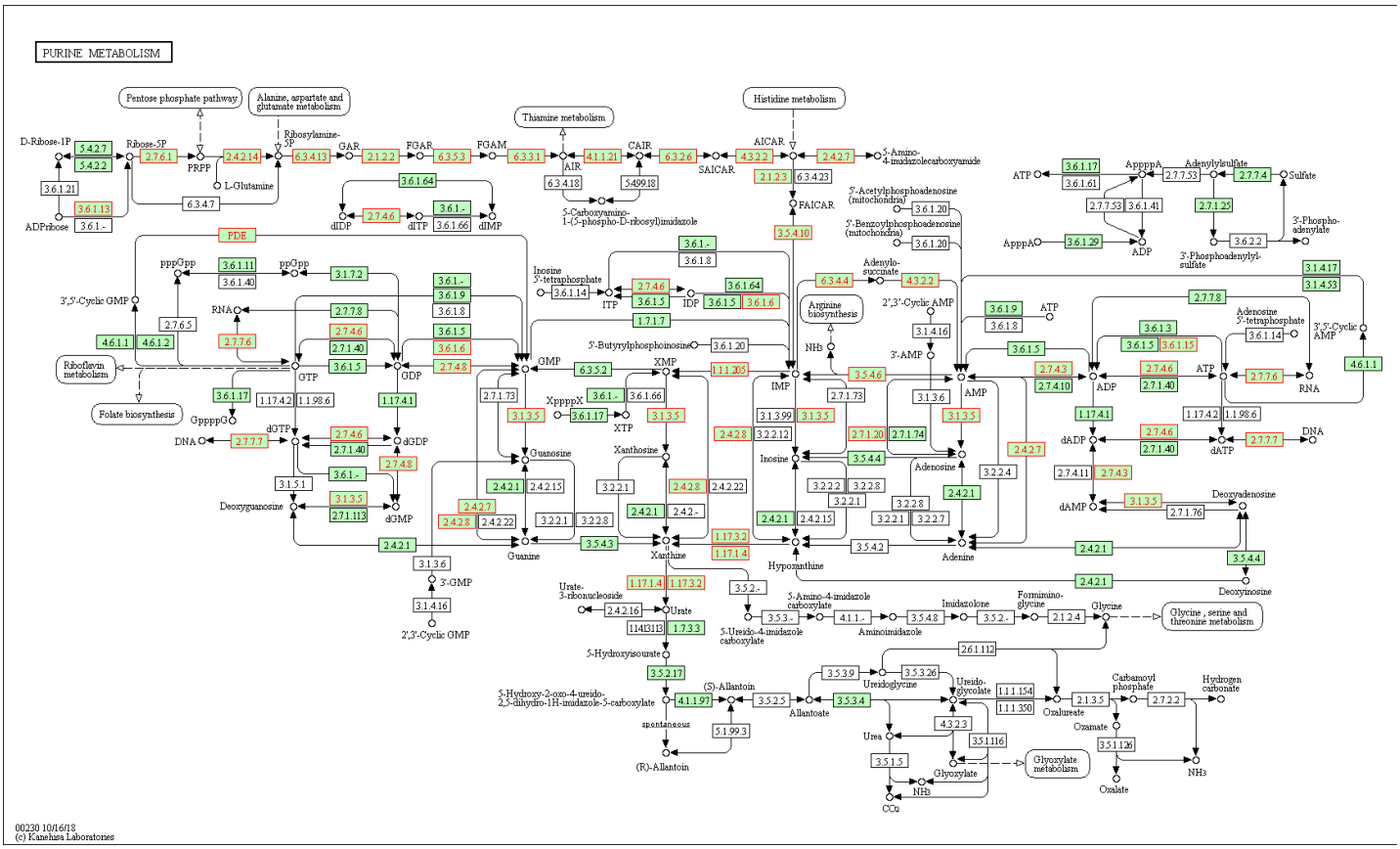
In turn, IFN-γ producers showed enrichment in metabolic pathways related with the biosynthesis of aminoacids and nucleotide metabolism such as purine (**Figure 4.2.21**) and pyrimidine metabolism (**Table 4.6** and **Table 4.8**). The data obtained by Gene Ontology term enrichment analysis together with the pathway enrichment analysis suggests that these cells are more active in processes related to genetic information processing like transcription (**Figure 4.2.22**), translation, replication and DNA repair. The enrichment in the machinery responsible for replication, transcription and translation might reflect different abilities regarding cell division/proliferation. The pyrimidine and purine metabolism pathways aforementioned fall also within this theme but there are other interesting pathways found enriched in the YFP$^+$ cells such as the pentose phosphate pathway, oxidative phosphorylation or thiamine metabolism, all of which constitute interesting findings.

Another aspect that might be different between these two populations is cell death. In terms of molecules responsible for inducing cell death in other cells, IL-17$^+$ $\gamma\delta$ **T** cells expressed ~6 times more *Gzmb* , whereas their IFN-γ^+ counterparts showed a ~5 fold enrichment for *Fasl*. Interestingly, IL-17$^+$ $\gamma\delta$ **T** cells expressed higher levels of Fas, the receptor for Fasl, together with other death receptor, *Tnfrsf10b* (DR5/Trailr2), possibly making them more susceptible to induction of apoptosis through death receptors. On a side note death receptors such as *Tnfrsf1a* (Tnfr1) and *Tnfrsf25* (DR3) were also more expressed in IL-17$^+$ cells, especially DR3 with a ~26 FC, but these are more likely to promote inflammation than cell death.

With regard to Natural Killer (NK) cell signatures, it is interesting to note that IL-17$^+$ $\gamma\delta$ **T** cells showed higher expression of the activating receptors ITGAL/ITGB2 and NKG2D, whereas IFN-γ^+ $\gamma\delta$ **T** cells were enriched in NKp46 and Ly49D activating

receptors, as well as inhibitory receptors such as Ly49A, Ly49C, Ly49G2, Ly49I and CD94/NKG2A/B.

Figure 4.2.21 - Purine metabolism (KEGG) for genes enriched in YFP⁺ γδ T cells.
Graphic representation of the position and interactions of the genes involved in the purine



metabolism with genes found more expressed in the IFN-γ⁺ cells labelled in red text. White rectangles represent genes that have no homology in mouse.

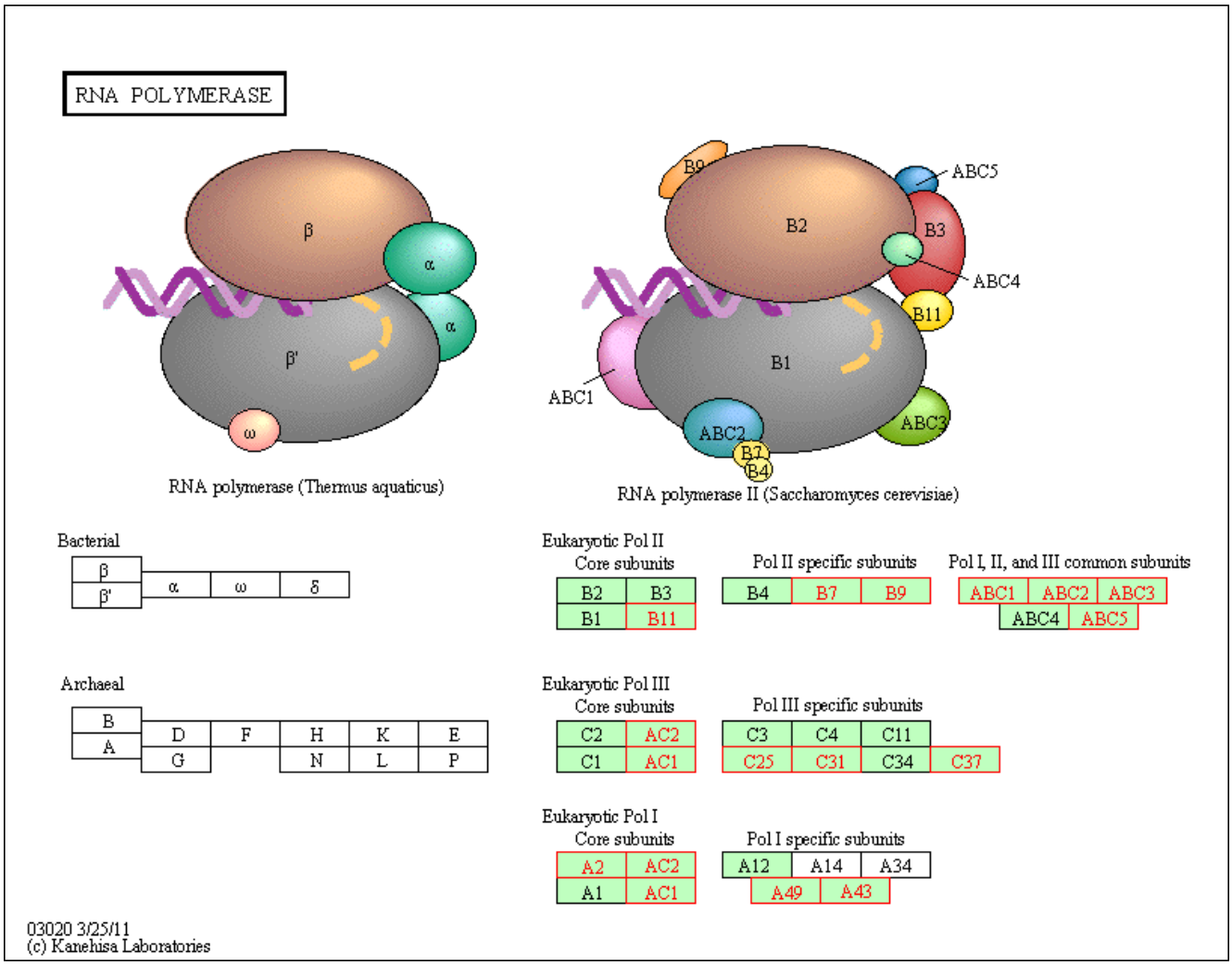


Figure 4.2.22 - RNA polymerase (KEGG) for genes enriched in YFP$^+$ γδ T cells.
Graphic representation of the position and interactions of the genes involved in RNA polymerase with genes found more expressed in the IFN-γ$^+$ γδ T cells labelled in red text. White rectangles represent genes that have no homology in mouse.

In conclusion, our analysis of the transcriptome revealed striking differences in what concerns the molecular pathways and cellular processes enriched in each of the effector γδ T cell subsets. Besides cytokine expression, our data suggests that IL-17-producing γδ T cells differ from their IFN-γ-producing counterparts in their ability to sense and integrate external cues, their metabolism, cell death pathways and the potential for replication, transcription and translation. These results highlight how IL-17 versus IFN-γ production is associated with substantially different cellular identities within the γδ T cell lineage.

Table 4.5 - KEGG pathways enriched in GFP⁺ γδ T cells.

Name	*KEGG class*	*Foreground*	*Background*	*p-value*
Protein processing in endoplasmic reticulum	Genetic Information Processing; Folding, sorting and degradation	67	166	1,15E-11
TNF signaling pathway	Environmental Information Processing; Signal transduction	46	108	3,50E-09
Apoptosis	Cellular Processes; Cell growth and death	51	136	5,82E-08
Th17 cell differentiation	Organismal Systems; Immune system	42	102	5,82E-08
Chemokine signaling pathway	Organismal Systems; Immune system	65	195	7,29E-08
Axon guidance	Organismal Systems; Development	59	175	2,26E-07
Regulation of actin cytoskeleton	Cellular Processes; Cell motility	66	210	6,20E-07
Lysosome	Cellular Processes; Transport and catabolism	45	124	9,78E-07
Focal adhesion	Cellular Processes; Cellular community - eukaryotes	61	199	4,40E-06
Th1 and Th2 cell differentiation	Organismal Systems; Immune system	34	87	4,80E-06
Osteoclast differentiation	Organismal Systems; Development	43	128	1,49E-05
NOD-like receptor signaling pathway	Organismal Systems; Immune system	52	168	1,83E-05
MAPK signaling pathway	Environmental Information Processing; Signal transduction	78	289	2,09E-05
T cell receptor signaling pathway	Organismal Systems; Immune system	36	103	3,25E-05
Platelet activation	Organismal Systems; Immune system	41	124	3,25E-05
Rap1 signaling pathway	Environmental Information Processing; Signal transduction	60	209	3,53E-05
Cytokine-cytokine receptor interaction	Environmental Information Processing; Signaling molecules and interaction	73	271	3,90E-05
Adherens junction	Cellular Processes; Cellular community - eukaryotes	27	72	9,60E-05
Phosphatidylinositol signaling system	Environmental Information Processing; Signal transduction	33	98	1,38E-04
Thyroid hormone signaling pathway	Organismal Systems; Endocrine system	37	115	1,38E-04
cAMP signaling pathway	Environmental Information Processing; Signal transduction	55	197	1,47E-04
FoxO signaling pathway	Environmental Information Processing; Signal transduction	40	132	2,50E-04
Leukocyte transendothelial migration	Organismal Systems; Immune system	36	115	2,80E-04
Endocytosis	Cellular Processes; Transport and catabolism	69	270	2,81E-04
Jak-STAT signaling pathway	Environmental Information Processing; Signal transduction	46	165	5,96E-04
ErbB signaling pathway	Environmental Information Processing; Signal transduction	28	85	6,72E-04
Cellular senescence	Cellular Processes; Cell growth and death	50	186	7,46E-04
Amino sugar and nucleotide sugar metabolism	Metabolism; Carbohydrate metabolism	19	49	7,46E-04
Sphingolipid signaling pathway	Environmental Information Processing; Signal transduction	36	122	8,58E-04
Relaxin signaling pathway	Organismal Systems; Endocrine system	38	131	8,58E-04
Inositol phosphate metabolism	Metabolism; Carbohydrate metabolism	24	72	1,44E-03
Toll-like receptor signaling pathway	Organismal Systems; Immune system	30	99	1,72E-03
Natural killer cell mediated cytotoxicity	Organismal Systems; Immune system	34	118	1,96E-03
IL-17 signaling pathway	Organismal Systems; Immune system	28	91	1,96E-03
VEGF signaling pathway	Environmental Information Processing; Signal transduction	20	58	2,55E-03
Insulin signaling pathway	Organismal Systems; Endocrine system	38	141	3,53E-03
NF-kappa B signaling pathway	Environmental Information Processing; Signal transduction	30	104	3,76E-03
Fc gamma R-mediated phagocytosis	Organismal Systems; Immune system	26	87	4,46E-03
Phagosome	Cellular Processes; Transport and catabolism	46	183	4,85E-03
Notch signaling pathway	Environmental Information Processing; Signal transduction	17	49	5,27E-03
N-Glycan biosynthesis	Metabolism; Glycan biosynthesis and metabolism	17	49	5,27E-03
Neurotrophin signaling pathway	Organismal Systems; Nervous system	33	121	5,43E-03
Gap junction	Cellular Processes; Cellular community - eukaryotes	25	86	7,61E-03
Sphingolipid metabolism	Metabolism; Lipid metabolism	16	48	1,08E-02
GnRH signaling pathway	Organismal Systems; Endocrine system	25	89	1,23E-02
B cell receptor signaling pathway	Organismal Systems; Immune system	21	72	1,50E-02
Estrogen signaling pathway	Organismal Systems; Endocrine system	26	96	1,63E-02
RIG-I-like receptor signaling pathway	Organismal Systems; Immune system	20	68	1,63E-02
HIF-1 signaling pathway	Environmental Information Processing; Signal transduction	28	106	1,71E-02
Ras signaling pathway	Environmental Information Processing; Signal transduction	53	233	1,77E-02
ECM-receptor interaction	Environmental Information Processing; Signaling molecules and interaction	23	83	1,88E-02
Cholinergic synapse	Organismal Systems; Nervous system	29	113	2,16E-02
Steroid biosynthesis	Metabolism; Lipid metabolism	8	19	2,30E-02
p53 signaling pathway	Cellular Processes; Cell growth and death	19	67	2,66E-02
Long-term potentiation	Organismal Systems; Nervous system	19	67	2,66E-02
Phospholipase D signaling pathway	Environmental Information Processing; Signal transduction	35	147	3,13E-02
Oxytocin signaling pathway	Organismal Systems; Endocrine system	36	153	3,37E-02
GABAergic synapse	Organismal Systems; Nervous system	23	88	3,42E-02
Cell adhesion molecules (CAMs)	Environmental Information Processing; Signaling molecules and interaction	39	169	3,47E-02
Glutamatergic synapse	Organismal Systems; Nervous system	28	114	3,95E-02
Calcium signaling pathway	Environmental Information Processing; Signal transduction	41	183	4,69E-02
Hippo signaling pathway - multiple species	Environmental Information Processing; Signal transduction	9	26	4,98E-02

Table 4.6 - KEGG pathways enriched in YFP⁺ γδ T cells

Name	*KEGG Class*	*Foreground*	*Background*	*p-value*
Ribosome	Genetic Information Processing; Translation	113	177	4,93E-56
DNA replication	Genetic Information Processing; Replication and repair	22	35	7,66E-10
RNA transport	Genetic Information Processing; Translation	54	167	2,39E-09
Spliceosome	Genetic Information Processing; Transcription	43	133	2,03E-07
Metabolic pathways	Metabolic pathways	233	1322	6,57E-07
Pyrimidine metabolism	Metabolism; Nucleotide metabolism	35	101	6,57E-07
Ribosome biogenesis in eukaryotes	Genetic Information Processing; Translation	38	116	6,57E-07
Purine metabolism	Metabolism; Nucleotide metabolism	50	178	1,10E-06
RNA polymerase	Genetic Information Processing; Transcription	15	30	2,63E-05
Oxidative phosphorylation	Metabolism; Energy metabolism	37	134	6,64E-05
Nucleotide excision repair	Genetic Information Processing; Replication and repair	18	44	6,64E-05
Cysteine and methionine metabolism	Metabolism; Amino acid metabolism	18	49	3,66E-04
Mismatch repair	Genetic Information Processing; Replication and repair	11	22	5,03E-04
Biosynthesis of amino acids	no class, "Global and overview maps"	23	77	9,69E-04
Antigen processing and presentation	Organismal Systems; Immune system	25	91	2,02E-03
Carbon metabolism	no clas, "Global and overview maps"	30	120	2,51E-03
Intestinal immune network for IgA production	Organismal Systems; Immune system	15	43	2,51E-03
Hematopoietic cell lineage	Organismal Systems; Immune system	25	95	3,50E-03
Th1 and Th2 cell differentiation	Organismal Systems; Immune system	23	87	5,45E-03
Cell cycle	Cellular Processes; Cell growth and death	29	124	9,09E-03
Base excision repair	Genetic Information Processing; Replication and repair	12	35	9,83E-03
Pentose phosphate pathway	Metabolism; Carbohydrate metabolism	11	32	1,29E-02
Thiamine metabolism	Metabolism; Metabolism of cofactors and vitamins	7	15	1,29E-02
RNA degradation	Genetic Information Processing; Folding, sorting and degradation	21	83	1,29E-02
Proteasome	Genetic Information Processing; Folding, sorting and degradation	14	46	1,29E-02
Natural killer cell mediated cytotoxicity	Organismal Systems; Immune system	27	118	1,41E-02
Cytosolic DNA-sensing pathway	Organismal Systems; Immune system	16	64	4,69E-02

Note: **Table 4.5** and **Table 4.6** contain the lists of KEGG pathways found to be enriched in GFP⁺ IL-17 expressing or YFP⁺ IFN-γ expressing γδ T cells, respectively. The KEGG class is also indicated as well as the number of the foreground and background genes along with the corresponding p-value. Entries are ordered from top to bottom by increasing p-value.

Table 4.7 - Reactome pathways enriched in GFP⁺ γδ T cells.

Name	*Foreground*	*Background*	*p-value*
Immune System	406	1655	4,59E-11
Asparagine N-linked glycosylation	93	274	5,59E-08
Vesicle-mediated transport	163	597	1,30E-06
Cytokine Signaling in Immune system	118	403	2,70E-06
Membrane Trafficking	153	562	3,20E-06
Rho GTPase cycle	52	138	6,50E-06
Innate Immune System	231	959	4,32E-05
Hemostasis	143	541	4,46E-05
Axon guidance	80	262	4,73E-05
Death Receptor Signalling	47	128	4,73E-05
Transport to the Golgi and subsequent modification	58	172	5,21E-05
COPI-mediated anterograde transport	34	85	1,75E-04
Platelet activation, signaling and aggregation	78	263	1,84E-04
Signaling by Interleukins	85	299	3,94E-04
Intra-Golgi and retrograde Golgi-to-ER traffic	57	180	4,66E-04
VEGFA-VEGFR2 Pathway	35	93	4,83E-04
Calnexin/calreticulin cycle	14	23	5,02E-04
Interleukin-2 family signaling	20	41	5,20E-04
ER to Golgi Anterograde Transport	47	142	6,79E-04
Interleukin-3, Interleukin-5 and GM-CSF signaling	20	42	7,25E-04
Toll Like Receptor 4 (TLR4) Cascade	38	107	7,25E-04
Signaling by VEGF	36	101	1,08E-03
Antigen Presentation: Folding, assembly and peptide loading of class I MHC	17	35	2,18E-03
Interleukin receptor SHC signaling	14	26	2,18E-03
N-glycan trimming in the ER and Calnexin/Calreticulin cycle	16	32	2,18E-03
TRIF(TICAM1)-mediated TLR4 signaling	32	89	2,18E-03
MyD88-independent TLR4 cascade	32	89	2,18E-03
Interferon Signaling	26	67	2,44E-03
Signaling by Rho GTPases	105	411	2,52E-03
ER Quality Control Compartment (ERQC)	11	18	2,90E-03
Sphingolipid metabolism	31	87	2,97E-03
Signaling by NOTCH	19	44	4,22E-03
Fcgamma receptor (FCGR) dependent phagocytosis	29	81	4,34E-03
Synthesis of PIPs at the plasma membrane	20	48	4,77E-03
Toll-like Receptor Cascades	45	147	4,93E-03
p75 NTR receptor-mediated signalling	30	86	5,09E-03
Semaphorin interactions	21	52	5,09E-03
FCERI mediated Ca+2 mobilization	13	26	6,58E-03
Neutrophil degranulation	126	525	6,58E-03
NRAGE signals death through JNK	21	53	6,58E-03
Integrin alphaIIb beta3 signaling	13	26	6,58E-03
ISG15 antiviral mechanism	13	26	6,58E-03
ER-Phagosome pathway	14	29	6,58E-03
L1CAM interactions	21	53	6,58E-03
Integrin signaling	13	26	6,58E-03
FCERI mediated MAPK activation	14	29	6,58E-03
Pre-NOTCH Processing in Golgi	6	7	6,66E-03
COPI-dependent Golgi-to-ER retrograde traffic	28	81	7,81E-03
Antiviral mechanism by IFN-stimulated genes	14	30	8,86E-03
PI Metabolism	26	74	9,24E-03
RIPK1-mediated regulated necrosis	9	15	1,01E-02
Regulated Necrosis	9	15	1,01E-02
Platelet Aggregation (Plug Formation)	15	34	1,07E-02
Cell death signalling via NRAGE, NRIF and NADE	25	71	1,07E-02
Adaptive Immune System	156	683	1,10E-02

Signaling by Receptor Tyrosine Kinases	96	392	1,39E-02
Interleukin-15 signaling	8	13	1,61E-02
G alpha (12/13) signalling events	26	77	1,61E-02
DDX58/IFIH1-mediated induction of interferon-alpha/beta	14	32	1,64E-02
Apoptotic cleavage of cellular proteins	16	39	1,64E-02
CRMPs in Sema3A signaling	9	16	1,66E-02
Clathrin-mediated endocytosis	39	133	2,02E-02
Glycosphingolipid metabolism	16	40	2,16E-02
Regulation of IFNG signaling	7	11	2,40E-02
Programmed Cell Death	31	100	2,40E-02
Caspase-mediated cleavage of cytoskeletal proteins	7	11	2,40E-02
Cell surface interactions at the vascular wall	34	113	2,41E-02
Unfolded Protein Response (UPR)	10	20	2,47E-02
Post-translational protein modification	276	1316	2,47E-02
Regulation of actin dynamics for phagocytic cup formation	21	60	2,57E-02
Interferon gamma signaling	9	17	2,57E-02
TNF signaling	16	41	2,60E-02
Class I MHC mediated antigen processing & presentation	87	360	2,98E-02
EPH-Ephrin signaling	24	73	3,10E-02
Non-integrin membrane-ECM interactions	10	21	3,42E-02
Signal transduction by L1	10	21	3,42E-02
Type I hemidesmosome assembly	6	9	3,42E-02
Interleukin-9 signaling	6	9	3,42E-02
Pre-NOTCH Expression and Processing	6	9	3,42E-02
TRIF-mediated programmed cell death	6	9	3,42E-02
Metabolism of lipids	147	661	3,57E-02
PECAM1 interactions	7	12	3,83E-02
Syndecan interactions	8	15	3,83E-02
Ligand-dependent caspase activation	8	15	3,83E-02
Interleukin-2 signaling	7	12	3,83E-02
Apoptotic execution phase	18	51	4,22E-02
Response to elevated platelet cytosolic Ca2+	39	141	4,73E-02
Downstream signal transduction	12	29	4,87E-02
Retrograde transport at the Trans-Golgi-Network	16	44	4,91E-02
Golgi-to-ER retrograde transport	33	115	4,91E-02
Regulation of gene expression by Hypoxia-inducible Factor	5	7	4,91E-02
Post-transcriptional silencing by small RNAs	5	7	4,91E-02
Phospholipid metabolism	50	192	4,91E-02

Note: List of Reactome pathways found to be enriched in GFP$^+$ IL-17 expressing $\gamma\delta$ T cells. The number of the foreground and background genes in indicated along with the corresponding p-value. Entries are ordered from top to bottom by increasing p-value.

Table 4.8 - Reactome pathways enriched in YFP$^+$ $\gamma\delta$ T cells.

Name	*Foreground*	*Background*	*p-value*
Translation	97	157	8,08E-44
Metabolism of RNA	177	483	4,66E-39
rRNA processing in the nucleus and cytosol	75	114	8,25E-37
Major pathway of rRNA processing in the nucleolus and cytosol	75	114	8,25E-37
rRNA processing	75	114	8,25E-37
Mitochondrial translation elongation	56	85	3,20E-27
Mitochondrial translation termination	56	87	1,70E-26
Mitochondrial translation	56	88	3,57E-26
Formation of a pool of free 40S subunits	35	41	3,05E-23
Formation of the ternary complex, and subsequently, the 43S complex	36	44	1,06E-22
Ribosomal scanning and start codon recognition	37	50	1,27E-20
L13a-mediated translational silencing of Ceruloplasmin expression	38	53	2,00E-20
Translation initiation complex formation	37	52	1,04E-19
Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S	37	53	2,80E-19
Eukaryotic Translation Initiation	39	60	1,18E-18
Cap-dependent Translation Initiation	38	59	5,63E-18
Synthesis of DNA	44	115	7,40E-10
Gap-filling DNA repair synthesis and ligation in TC-NER	30	62	1,43E-09
DNA Replication	45	123	2,30E-09
Transcription-Coupled Nucleotide Excision Repair (TC-NER)	33	75	3,22E-09
S Phase	49	143	4,20E-09
Processing of Capped Intron-Containing Pre-mRNA	66	229	1,21E-08
Nucleotide Excision Repair	39	107	4,59E-08
Dual incision in TC-NER	28	63	6,14E-08
The citric acid (TCA) cycle and respiratory electron transport	45	136	7,88E-08
Nucleotide metabolism	36	97	9,69E-08
Non-coding RNA Metabolism	23	46	9,69E-08
snRNP Assembly	23	46	9,69E-08
Mitotic G1-G1/S phases	42	129	4,18E-07
G1/S Transition	36	102	4,35E-07
DNA Replication Pre-Initiation	31	81	5,05E-07
pre-mRNA splicing	49	166	8,36E-07
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	31	83	9,35E-07
Cellular response to heat stress	31	84	1,30E-06
RNA Polymerase I Promoter Escape	16	28	2,10E-06
Cell Cycle	119	567	2,20E-06
Regulation of HSF1-mediated heat shock response	26	65	2,30E-06
Cell Cycle, Mitotic	107	497	2,60E-06
RNA Polymerase I Transcription Termination	16	29	3,60E-06
mRNA Splicing	49	175	4,20E-06
DNA Repair	70	288	4,60E-06
Nucleobase biosynthesis	11	15	6,00E-06
U12 Dependent Splicing	22	52	6,40E-06
Switching of origins to a post-replicative state	30	87	9,40E-06
RNA Polymerase I Transcription Initiation	19	42	1,20E-05
Activation of the pre-replicative complex	16	32	1,85E-05
Formation of TC-NER Pre-Incision Complex	21	51	1,95E-05
Cellular responses to stress	81	364	2,21E-05
Gene expression (Transcription)	177	969	2,81E-05
Assembly of the pre-replicative complex	24	65	3,16E-05
Glycolysis	23	63	6,53E-05
Orc1 removal from chromatin	24	68	7,76E-05
Carbohydrate metabolism	61	260	8,22E-05

Glucose metabolism	27	82	8,40E-05
Transport of Mature mRNA derived from an Intron-Containing Transcript	25	74	1,12E-04
Transport of Mature Transcript to Cytoplasm	25	74	1,12E-04
Recognition of DNA damage by PCNA-containing replication complex	14	29	1,33E-04
Metabolism	298	1826	1,45E-04
Extension of Telomeres	13	26	1,72E-04
Metabolism of proteins	269	1630	1,97E-04
Regulation of mRNA stability by proteins that bind AU-rich elements	26	82	2,47E-04
Downstream TCR signaling	27	87	2,57E-04
Gap-filling DNA repair synthesis and ligation in GG-NER	11	20	2,61E-04
RNA Pol II CTD phosphorylation and interaction with CE	13	27	2,70E-04
Cell Cycle Checkpoints	62	277	2,80E-04
Mismatch repair (MMR) directed by MSH2:MSH6 (MutSalpha)	9	14	2,94E-04
Telomere C-strand (Lagging Strand) Synthesis	12	24	3,48E-04
PCNA-Dependent Long Patch Base Excision Repair	11	21	4,42E-04
Complex I biogenesis	20	57	4,42E-04
Respiratory electron transport	21	62	5,02E-04
SUMOylation of RNA binding proteins	17	45	5,87E-04
Purine ribonucleoside monophosphate biosynthesis	8	12	5,87E-04
MMR	9	15	5,87E-04
mRNA Capping	13	29	6,13E-04
Activation of ATR in response to replication stress	15	37	6,21E-04
Resolution of Abasic Sites (AP sites)	15	37	6,21E-04
Base Excision Repair	15	37	6,21E-04
Regulation of Glucokinase by Glucokinase Regulatory Protein	13	30	8,92E-04
Termination of translesion DNA synthesis	13	30	8,92E-04
Global Genome Nucleotide Excision Repair (GG-NER)	24	79	9,06E-04
DNA Damage Bypass	17	47	1,01E-03
mRNA decay by 3' to 5' exoribonuclease	9	16	1,03E-03
Formation of ATP by chemiosmotic coupling	9	16	1,03E-03
Cristae formation	9	16	1,03E-03
Mismatch repair (MMR) directed by MSH2:MSH3 (MutSbeta)	7	10	1,03E-03
TCR signaling	29	105	1,03E-03
Dual Incision in GG-NER	15	39	1,14E-03
G2/M Checkpoints	38	154	1,19E-03
Formation of the Early Elongation Complex	13	31	1,20E-03
Cellular responses to external stimuli	83	425	1,59E-03
Mitochondrial biogenesis	11	24	1,59E-03
Resolution of AP sites via the multiple-nucleotide patch replacement pathway	11	24	1,59E-03
DNA strand elongation	11	24	1,59E-03
Association of TriC/CCT with target proteins during biosynthesis	6	8	1,91E-03
APC/C:Cdc20 mediated degradation of Securin	20	64	2,10E-03
SUMOylation of ubiquitinylation proteins	14	37	2,18E-03
Processive synthesis on the C-strand of the telomere	7	11	2,18E-03
SUMOylation of DNA damage response and repair proteins	22	74	2,18E-03
Translesion synthesis by Y family DNA polymerases bypasses lesions on DNA template	14	37	2,18E-03
SUMOylation of SUMOylation proteins	13	33	2,31E-03
Autodegradation of Cdh1 by Cdh1:APC/C	19	60	2,38E-03
Nuclear Pore Complex (NPC) Disassembly	13	34	3,23E-03
SCF-beta-TrCP mediated degradation of Emi1	17	52	3,32E-03
Transcriptional Regulation by TP53	57	274	3,32E-03
SUMOylation of DNA replication proteins	15	43	3,39E-03
Interconversion of nucleotide di- and triphosphates	11	26	3,39E-03
Translesion synthesis by POLI	8	15	3,47E-03
KSRP (KHSRP) binds and destabilizes mRNA	8	15	3,47E-03
Processive synthesis on the lagging strand	8	15	3,47E-03

Cyclin E associated events during G1/S transition	20	67	3,63E-03
Nuclear Envelope Breakdown	16	48	3,68E-03
Methionine salvage pathway	4	4	4,23E-03
Hypusinylation	4	4	4,23E-03
Regulation of APC/C activators between G1/S and early anaphase	22	79	5,22E-03
CDK-mediated phosphorylation and removal of Cdc6	20	69	5,22E-03
Cdc20:Phospho-APC/C mediated degradation of Cyclin A	20	69	5,22E-03
Cyclin A:Cdk2-associated events at S phase entry	20	69	5,22E-03
APC/C:Cdh1 mediated degradation of Cdc20 and other APC/C:Cdh1 targeted proteins in late mitosis/early G1	20	69	5,22E-03
Pentose phosphate pathway	8	16	5,63E-03
RNA Polymerase II Transcription	146	863	5,78E-03
Detoxification of Reactive Oxygen Species	12	32	5,88E-03
Formation of RNA Pol II elongation complex	17	55	5,94E-03
RNA Polymerase II Transcription Elongation	17	55	5,94E-03
TNFR2 non-canonical NF-kB pathway	25	96	6,04E-03
APC:Cdc20 mediated degradation of cell cycle proteins prior to satisfation of the cell cycle checkpoint	20	70	6,04E-03
Regulation of RAS by GAPs	19	65	6,04E-03
Synthesis of active ubiquitin: roles of E1 and E2 enzymes	11	28	6,07E-03
Lagging Strand Synthesis	9	20	6,07E-03
Amino acid and derivative metabolism	52	252	6,08E-03
Transcriptional Regulation by E2F6	10	24	6,12E-03
Sulfur amino acid metabolism	10	24	6,12E-03
CDT1 association with the CDC6:ORC:origin complex	17	56	6,95E-03
Gene Silencing by RNA	27	108	6,95E-03
TP53 Regulates Transcription of DNA Repair Genes	14	42	7,35E-03
Removal of the Flap Intermediate from the C-strand	6	10	7,71E-03
RNA Polymerase II Transcription Initiation And Promoter Clearance	15	47	7,71E-03
RNA Polymerase II Promoter Escape	15	47	7,71E-03
RNA Polymerase II Transcription Initiation	15	47	7,71E-03
RNA Polymerase II Transcription Pre-Initiation And Promoter Opening	15	47	7,71E-03
APC/C:Cdc20 mediated degradation of mitotic proteins	20	72	8,07E-03
SCF(Skp2)-mediated degradation of p27/p21	17	57	8,21E-03
Transcriptional regulation by small RNAs	25	99	8,76E-03
Metabolism of polyamines	22	83	8,79E-03
Ubiquitin-dependent degradation of Cyclin D	15	48	9,34E-03
Ubiquitin-dependent degradation of Cyclin D1	15	48	9,34E-03
Cross-presentation of soluble exogenous antigens (endosomes)	15	48	9,34E-03
Activation of APC/C and APC/C:Cdc20 mediated degradation of mitotic proteins	20	73	9,34E-03
Activation of NF-kappaB in B cells	18	63	9,56E-03
Stabilization of p53	16	53	9,56E-03
G1/S DNA Damage Checkpoints	18	63	9,56E-03
Mitotic Metaphase and Anaphase	38	174	9,62E-03
Translesion synthesis by REV1	7	14	1,03E-02
Removal of the Flap Intermediate	7	14	1,03E-02
Autodegradation of the E3 ubiquitin ligase COP1	15	49	1,10E-02
Ubiquitin Mediated Degradation of Phosphorylated Cdc25A	15	49	1,10E-02
Regulation of RUNX2 expression and activity	15	49	1,10E-02
p53-Independent DNA Damage Response	15	49	1,10E-02
p53-Independent G1/S DNA damage checkpoint	15	49	1,10E-02
Regulation of mitotic cell cycle	22	85	1,11E-02
Degradation of DVL	16	54	1,11E-02
APC/C-mediated degradation of cell cycle proteins	22	85	1,11E-02
M Phase	69	368	1,11E-02
Separation of Sister Chromatids	37	170	1,11E-02
SUMOylation of chromatin organization proteins	18	65	1,30E-02
RNA Polymerase III Transcription Initiation From Type 3 Promoter	10	27	1,44E-02

Mitotic Anaphase	37	173	1,51E-02
Methylation	7	15	1,55E-02
Translesion synthesis by POLK	7	15	1,55E-02
Phosphorylation of CD3 and TCR zeta chains	7	15	1,55E-02
p53-Dependent G1/S DNA damage checkpoint	17	61	1,56E-02
p53-Dependent G1 DNA Damage Response	17	61	1,56E-02
UCH proteinases	23	93	1,56E-02
Deadenylation-dependent mRNA decay	16	56	1,58E-02
Transcriptional regulation by RUNX3	20	77	1,60E-02
Downstream signaling events of B Cell Receptor (BCR)	20	77	1,60E-02
FCERI mediated NF-kB activation	19	72	1,68E-02
RNA Polymerase II Transcription Termination	17	62	1,82E-02
Cleavage of Growing Transcript in the Termination Region	17	62	1,82E-02
RNA Polymerase II Pre-transcription Events	20	78	1,85E-02
The role of GTSE1 in G2/M progression after G2 checkpoint	16	57	1,85E-02
FBXL7 down-regulates AURKA during mitotic entry and in early mitosis	15	52	1,87E-02
mitochondrial fatty acid beta-oxidation of unsaturated fatty acids	3	3	2,00E-02
Pyrimidine biosynthesis	3	3	2,00E-02
Translocation of ZAP-70 to Immunological synapse	6	12	2,02E-02
B Cell Activation	24	101	2,08E-02
Transcriptional regulation by RUNX2	16	58	2,18E-02
Mitotic G2-G2/M phases	36	172	2,28E-02
HDR through Homologous Recombination (HRR)	17	64	2,49E-02
Degradation of GLI1 by the proteasome	15	54	2,69E-02
Butyrate Response Factor 1 (BRF1) binds and destabilizes mRNA	7	17	3,26E-02
RNA Polymerase I Chain Elongation	20	82	3,26E-02
Regulation of RUNX3 expression and activity	14	50	3,32E-02
G2/M Transition	35	170	3,33E-02
RNA Polymerase III Transcription Initiation From Type 2 Promoter	9	26	3,56E-02
RNA Polymerase III Transcription Initiation From Type 1 Promoter	9	26	3,56E-02
RNA Polymerase I Transcription	23	100	3,70E-02
GLI3 is processed to GLI3R by the proteasome	15	56	3,73E-02
Dectin-1 mediated noncanonical NF-kB signaling	15	56	3,73E-02
NIK-->noncanonical NF-kB signaling	15	56	3,73E-02
Degradation of beta-catenin by the destruction complex	19	78	3,92E-02
Formation of Incision Complex in GG-NER	12	41	4,03E-02
Degradation of AXIN	14	52	4,63E-02
Oxygen-dependent proline hydroxylation of Hypoxia-inducible Factor Alpha	16	63	4,87E-02
CLEC7A (Dectin-1) signaling	21	91	4,91E-02

Note: List of Reactome pathways found to be enriched in YFP$^+$ IFN-γ expressing $\gamma\delta$ T cells. The number of the foreground and background genes in indicated along with the corresponding p-value. Entries are ordered from top to bottom by increasing p-value.

GENERAL DISCUSSION

5. General discussion

MicroRNAs are employed by cells to regulate and fine-tune gene expression. They act at the post-transcriptional level on messenger RNAs, inhibiting their translation or promoting their destabilization and consequent degradation [2]. MicroRNAs hold crucial functions and are essential for the efficient performance of many systems and cell types throughout animal classes, vertebrates or invertebrates, fungi and plants [2]. In mammals, they have been shown to be important within several body systems and cell types, such as both the myeloid and lymphoid lineages of the immune system, where, in general, they tend to act as a brake for effector functions. MicroRNAs have been implicated in the control of cytokine secretion, cell differentiation, proliferation and survival [37,224,225]. As such, they have also been implicated in diseases that are caused by deregulation of these processes [226,227]. The regulation of the production of pro-inflammatory cytokines, such as IFN-γ and IL-17, is essential for the maintenance of the host homeostasis. These two cytokines are essential to protect the host from pathogens like viruses, fungi and bacteria; however, excessive production of these cytokines is associated with chronic inflammation and autoimmune diseases, such as multiple sclerosis, type I diabetes, rheumatoid arthritis, psoriasis and colitis [228]. Different cells within the immune system have the ability to produce these cytokines. In a typical adaptive response to intracellular pathogens, the major sources of IFN-γ are $CD8^+$ T cells and their $CD4^+$ (Th1) counterparts, taking on from innate-like NK and $\gamma\delta$ T cells that provide most IFN-γ during the earlier stages.

It was within this framework that we decided to identify microRNAs regulating IFN-γ expression in $CD8^+$ T cells. The importance of microRNAs in this process was clearly demonstrated by overexpression of IFN-γ in thymic and peripheral Dicer-deficient $CD8^+$ T cells. By globally evaluating the expression of microRNAs in $CD8^+$ T cells expressing or not IFN-γ, we were able to identify 29 differentially expressed microRNAs from which we selected 6 to manipulate experimentally. We found that miR-181a-5p and miR-451a inhibit IFN-γ expression in $CD8^+$ T cells. We also found that miR-181a-5p was able to target a set of 3 mRNAs: *Id2*, *Akt2* and *Map2k1*. We further demonstrated that reducing *Id2* levels copied the phenotype observed when miR-181a-5p levels were increased. This validated our hypothesis that miR-181a-5p downregulates IFN-γ production in $CD8^+$ T

cells, at least in part, by targeting *Id2*. When present in low amounts, Id2 will not be able to prevent the binding of E2/E47 proteins to the upstream regions of the *Tbx21 locus*, which will lead to the silencing of this *locus* [183,187,229]. Unlike *Id2,* that besides having an extremely important role in $CD8^+$ T cell biology has also been biochemically-validated as a target of miR-181a-5p, the remaining 2 targets, *Akt2* and *Map2k1*, still need to be validated. Due to the nature and complexity behind the binding of microRNAs to their targets, software predicting this interaction generates false negatives and false positives. As such to understand which of the target genes are relevant one must do functional studies like the ones we presented above. In addition, to formally validate the interaction, one may use a luciferase assay, which consists in cloning the 3' UTR of the predicted target mRNA into a vector containing a luciferase reporter and introduce it into cells. Then, by experimentally increasing the levels of the microRNA, one can observe the impact on the expression of luciferase. By doing the same but with a 3' UTR where the binding region of the microRNA has been mutated and does not allow the interaction to occur, one can assess specificity. This is probably the most widely used method to validate a microRNA:mRNA interaction although it presents some caveats, such as the fact that it is usually performed in a cell line that will likely be very different, namely at the RNA landscape level, from the cells of interest ($CD8^+$ T cells in this case). With the recent advances in sequencing, other techniques have been brought forward, such as CLASH, that allows a direct identification of microRNA targets [230]. The basis of the CLASH technique is to capture the interaction between the microRNA and its target mRNA taking advantage of the fact that this occurs within the RISC. The first step consists on inducing, via UV exposure, a cross-link between the RNA molecules and the Ago protein in the complex. The second is to promote the ligation of the microRNA strand to the mRNA one, forming a hybrid strand. Following this, the complex is biochemically isolated, the hybrid RNA molecule extracted and sequenced. By bioinformatically analysing the sequences obtained it is possible to know which microRNAs were directly interacting with the transcript of which genes. This technique is very powerful since it is able to confirm the direct microRNA-target gene interaction but also provide some information on the motifs of the interaction. However, it also has some drawbacks: it is more expensive and requires a large numbers of the cells of interest, which might be a

decisive factor for people working with low frequency populations (such as γδ T cells in this thesis).

The fact that none of the studied targets of miR-451a showed a reduction upon transfection with the mimic tells us that miR-451a is impacting on IFN-γ expression via other different targets than the ones we anticipated, which still need to be identified. It is also interesting to note that both miR-181a-5p and miR-451a reduce IFN-γ production even though they were enriched in opposite populations, namely miR-181a-5p levels were higher in YFP^- cells while miR-451a was enriched in YFP^+ cells. Thus, miR-181a-5p seems to suppress the acquisition of IFN-γ expression, whereas miR-451a may prevent already differentiated cells to express too much of this cytokine, thereby fine tuning its expression in $CD8^+$ T cells.

The next step in our $CD8^+$ T cell project is to determine the impact that miR-181a and miR-451a have in $CD8^+$ T cell biology, such as IFN-γ production, in an *in vivo* context. To address this question we plan on using mouse strains where these microRNAs have been depleted. We have recently imported these two knock out (KO) mouse lines, which were kindly provided by Dr. Andreas Krueger (miR-181 KO) and Dr. Lily Huang (miR-451a KO) [215,231]. Upon establishment of the respective colonies, we plan to infect the KO mice (and littermate controls) with Murid herpesvirus-4 (MuHV-4), an infection model where $CD8^+$ T cells play a predominant role in resolving the infection. Following intranasal inoculation, MuHV-4 establishes a lytic infection in the respiratory track, mainly in lung alveolar epithelial cells, that peaks at around day 4-7 post-infection and is cleared by the immune system by day 10-12. $CD8^+$ CTLs are very important in the rapid control of the infection and are the major population detected in brochoalveolar lavages of infected animals. IFN-γ, as in the context of other viral infections, is one of the main cytokines produced in response to the virus and is critical for clearance of the pathogen [232–234]. Besides analysing the $CD8^+$ T cell response to MuHV-4, namely IFN-γ production and control of viral load, we will also assess the expression levels of the predicted targets of each microRNA in sorted $CD8^+$ T cells *ex vivo*. This will hopefully provide evidence that miR-181a-5p and miR-451a are part of the network that controls the expression of IFN-γ in $CD8^+$ T cells *in vivo*.

In the same way, we intend to make use of KO lines available (**Table 5.1**) to further study the microRNAs candidates that we have identified in *γδ* T cells, characterizing them in an *in vivo* context. As pointed out before, *γδ* T cells have two major functional subsets and participate in several types of the immune responses. As such, by using different models of disease, previously established in our lab, in KO versus WT (littermate) mice, we can determine what are the consequences the absence of a microRNA has on a specific *γδ* T cell effector population's response. For example, to test the impact of a microRNA on IL-17$^+$ cells we could use the Aldara cream (imiquimod)-induced psoriasis model [235]; on IFN-*γ*$^+$ cells the *Plasmodium berghei* [207] or MuHV-4 infection [236] models; and on IL-17$^+$ IFN-*γ*$^+$ cells the *Listeria monocytogenes* infection [211] or the intraperitoneal ID8 ovarian cancer cell model [68].

Table 5.1 - KO mice or ES (embryonic stem) cell resources for microRNA candidates identified in this study.

miRNA	KO	Type	KO details	Reference paper / Source
miR-7a	YES	Mouse	Cre-inducible. MiR-7a1 and MiR-7a2 conditional KO.	Latreille et al, (2014) J Clin Invest.
		ES cell	Whole animal. Reporter-tagged deletion allele.	EuMMCR or MMRRC.
miR-128	YES	Mouse	Cre-inducible. MiR-128-1 and miR-128-2 conditional KO.	Tan et al, (2013) Science.
		ES cell	Whole animal. Reporter-tagged deletion allele.	EuMMCR or MMRRC.
miR-139	YES	Mouse	Whole animal. Mir-139.	Mao et al, (2015) IJBCB.
miR-677	YES	ES cell	Whole animal. Reporter-tagged deletion allele.	EuMMCR or MMRRC.
miR-1949	YES	ES cell	Whole animal. Reporter-tagged deletion allele.	EuMMCR or MMRRC.
miR-322	YES	Mouse	Whole animal. KO for miR-322 but also for miR-424 and miR-503 that are in the proximity and are co-expressed together with miR-322.	Llobet-Navas et al, (2014) Genes Dev.
miR-326	YES	ES cell	Whole animal. Reporter-tagged deletion allele.	EuMMCR or MMRRC.
miR-450b	YES	ES cell	Whole animal. Reporter-tagged deletion allele.	EuMMCR or MMRRC.
miR-181	YES	Mouse	Whole animal. MiR-181a-1~ 181b-1, miR-181a-2~181b-2, miR-181c~181d.	Fragoso et al, (2012) PLoS Genet. Henao-Mejia et al, (2013) Immunity. Zietara et al, (2013) PNAS.
miR-451	YES	Mouse	Whole animal. MiR-144/miR-451a cluster depletion or only miR-451a depletion.	Rasmussen et al, (2010) JEM.

Our strategy to choose our microRNA candidates was a biased one, aimed at maximizing, in our view, the chances of finding microRNAs relevant for the biology of *γδ* T cells. Our logic to choose microRNAs that were highly enriched in an effector subset (e.g. IL-17

producers), and had predicted target genes important for the differentiation and function of other subset (e.g. IFN-γ program), relied on the assumption that microRNAs would be present to help maintain the current effector profile (IL-17$^+$) and act as a break to the acquisition of traits of the alternative fate (IFN-γ^+). As we saw in our CD8$^+$ T cell study, that may not be always the case; instead, microRNAs may be present to limit the excessive translation of effector mRNAs. This approach will generate similar networks to **Figure 4.2.13** that can also be very informative. In fact, as discussed in the previous section, some of the microRNAs that we selected that were enriched in YFP$^+$ $\gamma\delta$ T cells and had predicted targeted genes within the "IL-17 program" also presented predicted target genes within the "IFN-γ program"; and more strikingly impacted on IFN-γ but not IL-17 production. Other different reasonings can be applied that will most certainly lead to different microRNAs–target gene interaction networks, all based on the same data. That is one of the reasons why our initial miRNome characterization of IL-17$^+$, IFN-γ^+ and IL-17$^-$ IFN-γ^- $\gamma\delta$ T cells will be publicly available in the near future; and will constitute a new and valuable resource for the community interested in IL-17 or IFN-γ producing $\gamma\delta$ T cells to explore.

In a similar way, our transcriptome profiling data will also be made publicly available and will likely be of even more interest for the $\gamma\delta$ T cell community. Our analysis of this transcriptomic data (presented here) identified unanticipated differences between these two populations. Amongst them is the difference in ability of sensing extracellular cues, given by membrane receptors, together with the capacity to produce different chemotactic molecules that will recruit different cell types. Another very interesting finding was that the two subsets appear to rely differently on some metabolic pathways. Experiments measuring lipid uptake, likely to be higher in IL-17$^+$ cells based on our analysis, might reveal different requirements and changing lipid availability might result in changes in effector abilities. Another informative experiment would be to determine how these cells differ in terms of their ability to perform glycolysis and mitochondrial respiration, as our data suggest that IFN-γ^+ cells might be more active in this area. This could be done with a Seahorse XF that would also allow to measure the metabolic potential of these cells, that has been shown to be different between naïve and memory T cells for instance, and also

measure the amount of ATP coming either from the glycolytic pathway or the mitochondrial respiration pathway [237].

We hope the new knowledge presented here, and developed in the near future, may also be used in a context outside of basic research as manipulation of microRNAs may be used in a therapeutic context to adjust the immune cells' actions in a way that is beneficial for patients. This might include boosting an advantageous response or dampening the effects of a deleterious one. Therapeutically changing the levels of microRNAs may give us the ability to, for example, alter the capacity of immune cells to produce different types of effector molecules, such as the cytokines IFN-γ and IL-17. Given the serious consequences in human health that arise from the excessive release of either one of these cytokines, more knowledge about which microRNAs are involved in the regulatory circuits that govern their expression will only increase the chances to find one that can be used therapeutically. To increase this knowledge was precisely one of the purposes of the work described in this thesis. The identification of microRNA candidates, namely on corresponding human T cell subsets, constitutes a starting point in the effort to modulate them in T cells in order to mitigate the negative effects deregulated cytokine secretion has in patients. As discussed below, there are many hurdles and criteria that a candidate microRNA must successfully pass in order to be a good candidate for clinical application. However, with the growing amount of information on the mechanisms behind microRNAs' mode of action and due to the fact that microRNAs have an important role in inflammation and many diseases, including cancer, the scientific community has been investing in such an idea and several therapeutic approaches have emerged in the last years that aim at manipulating microRNA levels to improve certain pathologies, some already reaching the clinical trials phase.

There are already several studies reporting the impact that individual microRNAs in T cells have on inflammatory disorders. For example, human airway-infiltrating T cells from asthma patients have been shown to overexpress miR-19a, which promoted Th2 cytokine production, while conversely, its absence impaired Th2 cell responses [91]. On the other hand, miR-155 was found to be upregulated in inflammatory bowel disease (IBD), and miR-155$^{-/-}$ mice displayed lower clinical scores and reduced systemic and mucosal Th1 and Th17 cytokines in an experimental colitis model [238]. MiR-155 expression levels were also

elevated in $CD4^+$ T cells during experimental autoimmune encephalomyelitis (EAE), and miR-$155^{-/-}$ mice exhibited delayed course of disease and reduced inflammation in the CNS when compared with WT controls [136,239]. The attenuation of EAE in miR-155-deficient mice was shown to be T cell intrinsic and associated with a decrease in Th1 and Th17 type responses, directly correlated with decreased IFN-γ and IL-17 levels, respectively, in the CNS and in peripheral lymphoid organs [136,239]. MiR-155-deficient mice also fail to develop collagen-induced arthritis (CIA), which correlates with decreased levels of IL-17 and IL-22, two Th17 signature cytokines known to drive the disease development [240]. Moreover, diminished upregulation of miR-155 and miR-146a in response to T cell stimulation was observed in Treg cells from rheumatoid arthritis (RA) patients [241]. The decreased levels of miR-146a were correlated with increased expression levels of its direct target STAT1, a transcription factor known to drive IFN-γ expression, and were observed in patients with active disease, where Treg cells exhibited a pro-inflammatory phenotype [241]. Systemic lupus erythematosus (SLE) is another autoimmune disorder reported to display deregulated levels of certain microRNAs, including miR-31 and, again, miR-146a and miR-155, which are reduced in SLE patients compared to controls [242,243].

Amongst microRNA-based therapies under development we can find mirvirasen, a locked nucleic acid (LNA)-modified antisense inhibitor of miR-122, for the treatment of hepatitis C virus (HCV) infection. This strategy was born from the knowledge that miR-122 plays a very important role in increasing the stability and replication of the HVC's RNA genome, leading to a higher infection title [244–246]. Consistently, by inhibiting miR-122 using anti-miR-122 LNA molecules it was possible to reduce viral load and liver damage in mice and non-human primate models of HCV infection [247,248]. Using the same strategy it was possible to observe an encouraging prolonged dose-dependent reduction in the viral load in chronic HCV human patients with little adverse effects [249]. This strategy is now undergoing additional phase II clinical trials. RG-101 is also an anti-miR-122 that has passed phase I clinical trials and is now being studied under combination with antiviral agents [250].

RG-125 is a modified anti-miR-103/107 that is in stage I clinical trials for the treatment of patients with type 2 diabetes or non-alcoholic fatty liver disease (NAFLD). Non-

alcoholic steatohepatitis (NASH) is the most severe form of NAFLD, is often associated with insulin resistance and metabolic syndrome and is characterized by inflammation and abnormally high accumulation of fat in the liver that can progress over time into fibrosis which may lead to liver cirrhosis or cancer. The expression of miR-103/107 was found to be elevated in the livers of obese mice (both in diet induced or leptin deficient models) and of humans diagnosed with NASH [251]. Encouragingly in a mouse model of obesity with insulin resistance the treatment with a miR-103/107 inhibitor improved glucose homeostasis and insulin sensitivity. Amongst the known targets of miR-103/107 is Caveolin 1, a protein involved in insulin signalling [251].

Finally, in regard to cancer therapies there is one microRNA inhibitor and three microRNA mimics in different stages of clinical trials. Recently entered in phase I clinical trials are miR-155 inhibitor for cutaneous T cell lymphoma and mycosis fungoides, miR-29 mimic for scleroderma and miR-16 for malignant pleural mesothelioma or non cell small lung carcinoma [250,252]. After success in a tumour mouse model, a mimic for miR-34 was also entered in phase I clinical trials for the treatment of patients with several types of cancer (lymphoma, melanoma, primary liver cancer, small cell lung cancer, multiple myeloma or renal cell carcinoma) [250]. However, due to adverse side effects (lethal in some cases) caused by the immune system the trial was stopped. The mechanism behind this immune-related toxicity will have to be elucidated to allow for the design of new and safer trials [250].

There are several challenges to develop microRNA-mediated therapies. The first is to identify a microRNA, or a small set of, whose modulation will have a desired effect in a given pathology. The second is to ensure the stability and tissue specific targeting of the therapeutic agent, to make sure it acts mainly in the cells of interest and has reduced off-target effects in some other cell types that may uptake the oligonucleotide. One microRNA usually has the ability to target multiple genes, but the genes targeted depend on the cellular context and thus different pathways may be targeted by the same microRNA in different cell types or organs. This can lead to side effects or toxicity of the treatment. Development of novel delivery strategies that ensure a more efficient and stable delivery of

microRNA modulators will most certainly help in minimizing the off-target and toxicity potential inherent to this type of therapy.

When considering cancer this becomes a particularly difficult and complex challenge. Great heterogeneity can be found at the gene expression level amongst cancer cells within the same tumour and this heterogeneity is not static, as dynamic changes in the microenvironment, by hypoxia or inflammation for example, will also cause changes in the heterogeneity observed amongst cancer cells, making not only the microRNAs but also their mRNA targets change over time [253]. This not only makes it hard to identify microRNA candidates but also to make sure that the effect of therapeutic modulation introduced will be only beneficial and long-lasting. This is in addition to the impact genomic mutations or microenvironment changes can have on the biogenesis machinery of microRNAs itself. Many cancers show mutations in the core components of the pathway and, for example, hypoxia has been shown to downregulate Drosha and Dicer, deregulating the microRNA biogenesis and increasing tumour burden [254–258].

With all this in mind it is not unreasonable to argue that treatment with microRNA modulators will possibly be easier and more impactful in pathologies of organs or tissues more easily accessible and with less cellular heterogeneity, since the delivery will be more local and there will be less cell types where the introduction of the same microRNA mimic or inhibitor might lead to undesired side effects. The blood and liver are easily accessed via intravenous injection, as are the lungs and airways via aerosol and the skin topically. I believe these will be the settings where microRNA-mediated strategies will, more easily and strongly, show their true therapeutic potential. Although there are still no therapeutic microRNA candidates in clinical trials for lung pathologies either than cancer, this area also holds great potential, for example to minimize symptoms by restraining the action the immune cells in the case of allergies, asthma or autoimmune diseases. In fact it is precisely in this context, using microRNA modulators to control the inflammation or exacerbated immune responses, in the skin and airways of patients for example, that I believe the knowledge gathered on microRNAs in T cells will prove to be clinically most valuable.

Future research is needed and will undoubtedly bring us more information on the roles microRNAs are playing in immune cells, including in the control of cytokine expression. Candidates will then be used to test the impact their modulation can have in a disease context and hopefully will end up as being part of a successful therapy to help people and improve their lives.

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LIST OF PUBLISHED MANUSCRIPTS

7. List of published manuscripts

Tiago Amado, Nina Schmolka, Hozaifa Metwally, Bruno Silva-Santos, Anita Q. Gomes (2015). ***Cross-regulation between cytokine and microRNA pathways in T cells***. European Journal of Immunology.

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, Karine Serre, Amy H. Buck, Anita Q. Gomes, Bruno Silva-Santos (2018). ***MicroRNA-146a controls functional plasticity in γδ T cells by targeting NOD1***. Science Immunology.

Daniel P. Inácio, Tiago Amado, Bruno Silva-Santos, Anita Q. Gomes (2018). ***Control of T cell effector functions by miRNAs***. Cancer Letters.

Likai Tan, Inga Sandrock, Ivan Odak, Yuval Aizenbud, Anneke Wilharm, Joana Barros-Martins, Yaara Tabib, Alina Borchers, Tiago Amado, Lahiru Gongoda, Marco J Herold, Marc Schmidt-Supprian, Jan Kisielow, Bruno Silva-Santos, Christian Koenecke, Avi-Hai Hovav, Christian Krebs, Immo Prinz, Sarina Ravens (2019). ***Single-cell transcriptomics identifies the adaptation of Scart1^{+} Vγ6^{+} T cells to skin residency as activated effector cells***. Cell Reports.

Tiago Amado, Ana Amorim, Francisco J. Enguita, Paula V. Romero, Daniel Inácio, Marta Pires de Miranda, Samantha J. Winter, J. Pedro Simas, Andreas Krueger, Nina Schmolka, Bruno Silva-Santos, Anita Q. Gomes (2019). ***MicroRNA 181a regulates IFN-γ expression in effector CD8^{+} T cell differentiation***. Journal of Molecular Medicine.