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Functional impact of specific miRNAs on γδ T cell development

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for my godmother, Glória

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Summary (English)

γδ T cells are key providers of proinflammatory cytokines in various contexts of (patho)physiology. They are pre-programmed in the thymus into distinct subsets producing either interleukin-17 (IL-17) or interferon-γ (IFN-γ), which segregate with CD27 expression. In the periphery, $CD27^-$ γδ T cells, which usually express IL-17, can be induced to coexpress IL-17 and IFN-γ under highly inflammatory conditions. We have previously found that miR-146a was selectively enriched in these cells and restricted their IFN-γ production by targeting *Nod1* mRNA. In this project we aimed at further dissecting microRNA (miRNA)-mediated regulation of effector γδ T cell differentiation independently of the use of surface markers, which do not allow the isolation of pure populations of $IL\text{-}17^+$ or $IFN\text{-}\gamma^+$ γδ T cells. Instead, such pure γδ T cell populations were isolated from the peripheral lymphoid organs of a double reporter IL-17-GFP:IFN-γ-YFP mouse strain and subjected to next generation sequencing analysis of both microRNA and mRNA repertoires, which allowed for the first time the identification of miRNA and mRNA signatures directly associated with cytokine expression. Differentially expressed miRNAs and mRNAs were bioinformatically integrated into networks that allowed the prediction of respectively 6 and 3 miRNAs targeting key determinants of the IL-17 and IFN-γ programs of γδ T cells. In this work, we have validated by real-time quantitative PCR the expression pattern of these miRNAs in the corresponding foetal thymic γδ T cell subsets. Gain-of-function studies based on retroviral transduction of foetal liver progenitor cells co-cultured on OP9-DL1 stromal cells indicated that miR-326 and miR-450b regulate γδ T cell development, namely by inhibiting IFN-γ production. Moreover, both miRNAs levels specifically increased in γδ T cells that pre-commit to the IL-17 pathway but not to the IFN-γ pathway. Overall, by revealing two novel miRNAs with a role in γδ T cell thymic pre-commitment, the data described here support the importance of the thymic pre-programming of γδ T cell development and adds to the epigenetic knowledge of the regulation of γδ T cell effector function.

Keywords: miRNAs; γδ T cells; thymic development; T cell differentiation; cytokines.

Resumo (Portuguese)

O sistema imune é composto por uma variedade de moléculas e células efetoras que protegem o organismo do potencial dano causado por agentes infecciosos e pelas suas toxinas. Entre os vários componentes do sistema imune, os linfócitos apresentam o maior potencial no reconhecimento e destruição de microorganismos e são parte integral da imunidade adaptativa, sendo dotados da especificidade e memória imunológica que lhe são características. Os linfócitos T em particular são responsáveis pela imunidade celular, participando nomeadamente na resposta contra células infectadas ou cancerígenas. Dentro destes, as células T γδ constituem uma população conservada, crucial na produção de citocinas pró-inflamatórias em contextos de (pato)fisiologia, tais como a infecção e o cancro mas também em autoimunidade e homeostase. As células T γδ são pré-programadas no timo em dois subtipos que se distinguem pela produção de interleucina (IL)-17 ou interferão (IFN)-γ, segregados pela expressão do marcador de superfície CD27, e a sua migração do timo para órgãos linfoides e outros tecidos específicos como a derme tem início em ondas de subpopulações distintas ainda na vida embrionária Enquanto as células T γδ produtoras de IFN-γ são essenciais na resposta a infecções intracelulares, como *Listeria monocytogenes*, as células T γδ produtoras de IL-17 participam na resposta a patogénios extracelulares, como *Staphylococcus aureus* e *Candida albicans*. Adicionalmente ao seu papel protetor, estas duas subpopulações participam também em doenças autoimunes, como a esclerose múltipla ou a psoríase. Embora ainda não se conheçam os mecanismos subjacentes à pré-programação da função efetora de células T γδ no timo, estes estão na base da sua participação não-redundante na resposta inflamatória, especialmente na fase inicial, uma vez que lhes permite uma rápida secreção de citocinas após ativação por um estímulo.

Os microRNAs (miRNAs) são pequenas moléculas de RNA transcritos a partir do genoma cuja forma madura resulta do processamento pela maquinaria celular no núcleo e no citoplasma. Esta classe de moléculas de RNA não codificante silencia a expressão de genes alvo após emparelhamento com a região 3' UTR do respetivo RNA mensageiro (mRNA), promovendo a desestabilização dos transcritos ou a inibição da sua tradução. Como um miRNA pode interagir com vários mRNAs e levar à sua inibição, estima-se que mais de metade de todos os genes codificantes de proteínas em mamíferos estejam sujeitos a regulação por miRNAs. A sua contribuição na regulação de redes de expressão génica enquanto reguladores pós-transcricionais tem sido implicada em processos celulares tão fundamentais como a apoptose, o metabolismo, a divisão celular e a diferenciação celular, onde se inclui a diferenciação de linfócitos T. Além da participação crucial em homeostase, a desregulação dos níveis de expressão de miRNAs específicos em determinados tipos celulares tem sido associada a diversas condições patológicas, como as doenças autoimunes e o cancro.

Embora vários miRNAs tenham até ao momento sido implicados no controlo da diferenciação e função de muitos tipos de células do sistema imune, nomeadamente na regulação da produção de IFN-γ e IL-17 por outros linfócitos T, pouco se sabe sobre o seu papel nas células T γδ. O nosso grupo descreveu pela primeira vez o papel de um miRNA na regulação da função efetora e plasticidade das células T γδ. Demonstrou-se que as células T γδ $CD27^-$, que geralmente expressam IL-17 mas cuja co-expressão de IFN-γ pode ser induzida em condições inflamatórias como o microambiente tumoral, expressam seletivamente o miR-146a, que inibe a sua produção de IFN-γ através da sua ligação específica à região 3' UTR do mRNA-alvo *Nod1*.

Tendo em vista o estudo detalhado da regulação da diferenciação de células T γδ efetoras por miRNAs, explorámos uma estratégia independente do uso de marcadores de superfície, que não permitem o isolamento de populações puras de células T γδ produtoras de IL-17 ou IFN-γ. Assim, recorreu-se a uma estirpe de murganho duplo repórter para IL-17 (GFP) e IFN-γ (YFP), a partir da qual foram isoladas populações puras de células T γδ produtoras de IL-17 ou IFN-γ dos órgãos linfóides periféricos tendo os repertórios de miRNA e mRNA sido analisados por NGS "next-generation-sequencing". Os miRNAs e mRNAs diferencialmente expressos nas duas populações foram integrados bioinformaticamente em redes que permitiram a previsão de 6 miRNAs - miR-7a-5p, miR-92a-1-5p, miR-128-3p, miR-139-5p, miR-677-3p and miR-1949 que reprimem o programa de expressão de IL-17 e 3 miRNAs - miR-322-5p, miR-326-3p and miR-450b-3p – que reprimem o programa de produção de IFN-γ das células T γδ por interação com mRNAs-chave das suas redes de expressão génica.

O objectivo desta tese consistiu em analisar os potenciais efeitos destes miRNAs no desenvolvimento das células T γδ em termos da pré-programação no timo conducente à expressão de IL-17 ou IFN-γ. Confirmou-se que os miRNAs previamente selecionados a partir de células T γδ da periferia seguem o mesmo padrão de expressão diferencial nas populações correspondentes de células T γδ no timo fetal pré-programadas para a expressão de IL-17 ou IFN-γ, sugerindo que as assinaturas observadas na periferia são estabelecidas ainda durante o seu desenvolvimento no timo. De seguida, com vista à modulação funcional dos níveis dos miRNAs candidatos, procedeu-se à sua clonagem em retrovírus e estabeleceu-se um modelo de desenvolvimento de células T γδ que permite a modelação dos seus níveis de expressão através da transdução de células progenitoras de fígado fetal em co-cultura com células estromais OP9-DL1. Dado que, mesmo após uma extensa optimização em termos de combinações de citocinas adicionadas em pontos diferentes das co-culturas, não se conseguiram obter células T γδ produtoras de IL-17, que poderá ser uma limitação do sistema, as análises subsequentes foram focadas nos miRNAs envolvidos no programa de expressão de IFN-γ. A sua sobre-expressão nos progenitores em co-cultura com células OP9-DL1 mostrou que o miR-326 e o miR-450b regulam potencialmente o desenvolvimento de células T γδ, através da inibição da produção de IFN-γ. De acordo com esta observação, os níveis de expressão *ex vivo* de ambos os miRNAs aumentam especificamente em células que no timo fetal entram na via de expressão de IL-17 mas não em células que iniciam a pré-programação para produção de IFN-γ, sugerindo que, à semelhança do miR-146a, tanto o miR-326 como o miR-450b podem diminuir a plasticidade de células T γδ produtoras de IL-17, reprimindo a co-expressão de IFN-γ e mantendo a sua identidade celular. Mecanisticamente, previu-se que o miR-450b inibisse diretamente o mRNA do IFN-γ ligando-se à sua região 3' UTR, enquanto o miR-326 inibiria os mRNAs codificantes de três proteínas associadas à produção de IFN-γ, o marcador extracelular CD27 e oa factores de transcrição Egr2 e Eomesodermin (Eomes).

Experiências futuras incidirão numa caracterização funcional mais abrangente do efeito dos miRNAs na identidade e função das células T efectoras, bem como na modelação selectiva da produção de IL-17 e IFN-γ por células T γδ pela manipulação dos níveis de miRNAs. Os resultados obtidos poderão revelar interesse terapêutico em patologias associadas não só à desregulação da expressão de miRNAs nestas células mas também à desregulação da sua produção de citocinas.

No seu conjunto, os dados descritos nesta tese confirmam a importância da pré-programação que ocorre no timo durante o desenvolvimento de células T γδ e a manutenção da sua assinatura genética na periferia, e revelam dois novos miRNAs que desempenham um papel na regulação da pré-programação funcional de células T γδ, contribuindo para o conhecimento dos mecanismos epigenéticos subjacentes ao controlo da sua função efetora.

Palavras-chave: miRNAs; células T γδ; desenvolvimento de linfócitos T; timo; citocinas.

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Abbreviations[1]

CD	Cluster of differentiation
cDNA	Complementary DNA
D	Day
DGCR8	DiGeorge syndrome critical region gene 8
DN	Double negative
E	Embryonic day
EAE	Experimental autoimmune disease
Eomes	Eomesodermin
FACS	Fluorescence-activated cell sorting
FL	Foetal liver
FTOC	Foetal thymic organ cultures
IL	Interleukin
IFN	Interferon
MACS	Magnetic-activated cell sorting
MHC	Major histocompability complex
miR	microRNA
miRNA	microRNA
OP9-DL1	Recombination activating gene
RAG	Delta-like 1-expressing OP9 cell
RT-qPCR	Real-time quantitative polymerase chain reaction
RV	Retrovirus
TCR	T-cell receptor
Th	T helper cell
Treg	Regulatory T cell

[1] List of all abbreviations used at least two times throughout the manuscript. Additional abbreviations are defined in the text once they are first introduced to the reader.

1. Introduction

1.1. T cells within the adaptive immune system

The immune system is composed by a variety of molecules and effector cells that together protect the organism from the potential harm caused by infectious agents and their toxins. Among the components of the immune system, lymphocytes have the most powerful ability to recognize and target pathogenic microorganisms and they account for the whole of the adaptive compartment of the immune system, as opposed to the innate compartment, which functions as the first line of defence[1]. These lymphocytes express on their surface a unique antigen receptor among a highly variable repertoire that is highly diverse in its antigen-binding sites and are responsible for the high antigen specificity and immunological memory characteristic to the adaptive compartment[1].

In the vertebrate immune system, there are two major types of adaptive lymphocytes: B cells and T cells, distinguished by the structure of their antigen receptor. The B-cell receptor (BCR) is synthesised from the same genes as immunoglobulins, also known as antibodies and produced by these cells, while the T-cell receptor (TCR) is related to them but distinct in its structure and recognition properties[2]. The chromosomal organization of the genes that encode them is highly conserved among species and their variable antigen-recognition domains are encoded by exons which are assembled in developing T and B lymphocytes from scattered germline variable (V), diversity (D), and joining (J) gene segments through recombination activating gene (RAG)-mediated somatic rearrangement[2,3]. Besides the multiple combinations of possible joining events through V(D)J recombination, the generation of diverse antigen receptor repertoires is further enhanced by the addition of non-templated nucleotides to processed coding ends by the enzyme terminal deoxynucleotidyl transferase (TDT) in the final phase of the joining reaction[3].

Before the interaction of an antigen with an antigen receptor, lymphocytes are in an inactive state with little functional activity and are known as naïve lymphocytes; however, if exposed to their antigen they become activated as result of the interaction, and differentiate further into fully functional cells known as effector lymphocytes[4]. Some of these activated B and T lymphocytes differentiate into memory cells, granting the organism long-lasting immunity against a subsequent exposure to the same pathogen, characterized by an immediate and stronger response possible through the differentiation of these cells into effector cells[4].

Unlike B cells that develop in the bone marrow and are part of the humoral immunity, T cells develop in the thymus and are responsible for cellular immunity, namely the response to infected or transformed cells[1]. Focusing on T cells, it is during development in the thymus that these cells acquire the TCR, which then undergoes key selection processes to attest productive somatic rearrangement (positive selection) while avoiding self-reactivity (negative selection)[5]. For most T cells, this is the result of molecular interactions between the TCR and peptide-major histocompatibility complex (MHC) complexes[5], although for the minor subset of Natural Killer (NK)-like NKT cells the selecting element is CD1d presenting glycolipids[6]. For conventional T cells, the outcome of the preferential TCR binding to MHC class I or II is the selection into the $CD8^+$ or $CD4^+$ T cell lineages, respectively. $CD8^+$ T cells leave the thymus to become cytotoxic T lymphocytes (CTLs) upon cognate antigen recognition in the periphery[7]. Most $CD4^+$ T cells retain a naïve phenotype in the thymus, and will only differentiate into T helper (Th) cells upon activation in secondary lymphoid organs; however, 5-10% of $CD4^+$ thymocytes undergo commitment to the regulatory T (Treg) cell lineage, characterized by the expression of the master transcription factor Foxp3[8]. The peripheral differentiation of T cells relies on the selective expression of other lineage-defining master regulators that orchestrate unique transcriptional programs allowing the different T cell populations to secrete distinct sets of cytokines and other effector molecules[8]. The balance between distinct T cell subsets impacts on the regulation of

inflammation and autoimmunity. For example, whereas interferon (IFN)-γ-producing Th1 and interleukin (IL)-17-producing Th17 are pro-inflammatory T cells that can be beneficial in a context of infection but detrimental in chronic inflammation and autoimmune disease[9], the anti-inflammatory functions of Treg cells have a protective role in chronic inflammation and autoimmunity[10].

1.2. γδ T cells

The genes that make up the TCR span four genomic loci that translate into four protein chains that associate in two different heterodimers, α:β or γ:δ, from which only one is present in a given cell to form its characteristic TCR[11]. Instead of the α:β heterodimers that compose most of the TCR repertoire, a minor population of T cells express γ:δ heterodimers and therefore are known as γδ T cells[12].

γδ T cells are a unique and conserved population of T lymphocytes with fundamental and non-redundant contributions to several (patho)physiological settings, such as tissue homeostasis, infection, autoimmunity and cancer[12,13]. Such unique contributions from γδ T cells within T lymphocytes rely on several properties that collectively distinguish γδ T cells from αβ T cells: a distinct set of TCR-recognized antigens, distinctive kinetics in the immune response, unique functional potentials, particular suitability to confer protection in specific anatomical locations, primary value in young animals and critical responses to specific pathogens[12]. These properties will be addressed in more detail in the following paragraphs.

For instance, the TCRγδ seems to recognize its antigens directly without them being presented by the TCRαβ-interacting MHC complexes, which probably renders them the ability of recognizing and responding rapidly to molecules expressed by many different cell types[12].

The majority of γδ T cells present in the murine body arise during embryonic development and early neonatal period in waves of distinct cell subsets expressing different V_γ and V_δ gene segments and migrate to characteristic body locations (Table 1.1.)[14]. For example, while γδ T cells from the first wave to leave the thymus, which home to the epidermis and are known as dendritic epidermal γδ T cells (DETCs), are programmed to express IFN-γ and have a characteristic $V_\gamma5$ chain, γδ T cells from the second wave home to the mucosal epithelia of tissues such as the reproductive tract, lung and the dermis of the skin, and are programmed to secrete IL-17 when stimulated, with a TCR composed mainly of the $V_\gamma6$ gene segment[14]. This particular capability of colonizing secondary tissues with an already established effector function is due to a 'developmental pre-programming' during γδ T cell development in the thymus[15], which will be addressed in the next section.

Table 1.1. – Development, homing and cytokine profiles of mouse γδ T cell subsets.

Subset	Thymic development	Tissue distribution	Cytokines (and markers)
$V_\gamma1^+$	Embryonic and postnatal (E18 and beyond)	Lymphoid tissue, liver, lung	IFN-γ ($CD27^+CD122^+NK1.1^+$) (minor producers of IL-17)
$V_\gamma4^+$	Embryonic and postnatal (E16 and beyond)	Lymphoid tissue, lung, liver, dermis	IL-17 ($CD27^-CCR6^+$) IFN-γ ($CD27^+CD122^+$)
$V_\gamma5^+$	Exclusively embryonic (E13-E16)	Epidermis	IFN-γ ($CD27^+NK1.1^+$)
$V_\gamma6^+$	Exclusively embryonic (E14-E20)	Uterus, lung, tongue, liver, placenta, kidney	IL-17 ($CD27^-CCR6^+$)
$V_\gamma7^+$	Embryonic and postnatal (E18 and beyond)	Intestinal intraepithelial layer	IFN-γ ($CD27^+CD122^+$)

'Markers' refers to the cell-surface phenotype associated with the cytokine-production profile. Adapted from Papotto *et al.*, *Nat. Immunol.* 18, 604-611 (2017)[13].

1.2.1. Development of γδ T Cells

T cells develop and mature in the thymus, an organ formed by a network of epithelial cells known as the thymic stroma, which provides a unique microenvironment for T-cell development. The thymic stroma consists of several lobules, each composed of an outer cortical region, the thymic cortex, and an inner medulla[16]. T cells are generated from some of the progenitor cells present in the bone marrow which also give rise to B cells that migrate to the thymus and commit to the T-cell lineage through the signal transduction induced by the interaction of the Notch1 receptor with ligands expressed by thymic epithelial cells, initiating a T-cell specific gene expression program in the thymic progenitor cells[17,18]. Developing thymocytes pass then through a series of distinct stages marked by changes in the expression of the TCR and of cell-surface proteins, reflecting the state of functional maturation of the cell[19].

T cells differentiate in the thymus from a common $CD4^-CD8^-$ double negative (DN) progenitor, which after expansion rearranges the δ, γ and β chains of its TCR in the DN2-DN3 stage, from which γδ T cells diverge from the DN4 progressing-αβ T cells[20]. This progression through DN stage is characterized by changes in the expression of the activation and memory marker CD44 and of T cell-activation marker CD25[19]. Following γδ T cell lineage commitment, γδ T cells are thought to diverge into distinct functional subpopulations, with TCRγδ signalling playing an important role in the thymic differentiation of γδ T cells with distinct effector functions. For instance, 'strong' ligand-dependent signalling through the TCRγδ prohibits the development of IL-17-secreting γδ T cells, promoting instead commitment to an IFN-γ secreting fate[21–25].

The master transcription regulators of IL-17 and IFN-γ expression in γδ T cells are ROR-γt and T-bet, respectively, with both being part of distinct transcriptional networks driving the cells towards one of the two effector fates[15]. As for the IFN-γ pathway, strong TCRγδ signalling appears to be required for the establishment of a transcription factor network involving Egr2 and Egr3, which supresses ROR-γt and the IL-17 pathway[22,23,26]. Besides, both proteins are transcription regulators of Id3, which is essential for the differentiation of IFN-γ-producing γδ T cells. TCF1 and Lef1 are two additional transcription factors that act as downstream effectors of the canonical Wnt signalling pathway and promote the development of IFN-γ secreting γδ thymocytes[27].

Less is known regarding the transcriptional network driving IL-17 commitment by γδ T cells: besides Sox4 and Sox13, which are expressed before TCR signalling and induce ROR-γt expression[27], Notch signalling and its downstream effector Hes-1 have also been implied in the development of these cells[28]. Unexpectedly, the development of IL-17 pre-committed γδ T cells is believed to be restricted to foetal and perinatal life, as indicated by the failure of adult bone marrow transplantation to generate these cells, or the induction of activity of RAG1 after birth[29]. IL-17 biased γδ T cells are thus thought to be generated only in the foetal and neonatal thymus and to then persist as self-renewing and long-lived cells in the thymus and peripheral organs[29,30].

Although the mechanisms behind γδ T cell thymic commitment to a specific effector function are overall still unclear, two distinct γδ T cell developmental pathways that diverge from a common $CD24^-CD44^-CD45RB^-$ phenotype and allow the identification of γδ T cells pre-committed to the secretion of IL-17A or IFN-γ based on the expression of the extracellular markers CD44 and CD45RB have recently been described (Figure 1.1.), correlating with the V_γ-subsets previously mentioned[24] (Table 1.1.).

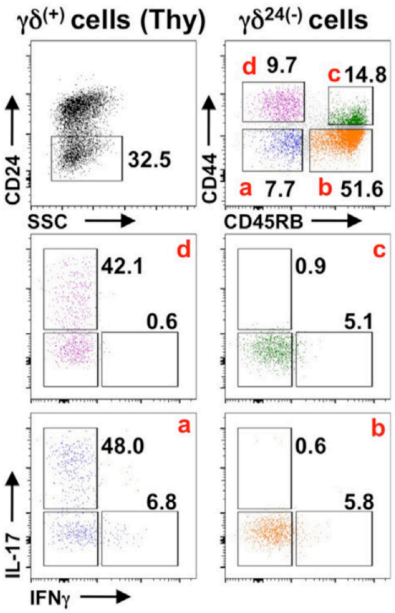

Figure 1.1. CD24, CD44, and CD45RB identify functionally distinct γδ T cell subsets. γδ T cells from (E17) thymic lobes. CD24$^-$ γδ T cells (TCRδ$^+$CD3ε$^+$) from top are sub-divided by CD44 and CD45RB (top right; subsets a-d). Middle and bottom plots show intracellular staining for IL-17A/IFN-γ in subsets a-d. From Sumaria *et al.*, *Cell Rep.* 19, 2469-2476 (2017)[17].

Following development and maturation in the thymus, many γδ T cells migrate directly to tissues, while some home to the lymph nodes[12] (Table 1.1.). Already in the periphery, they can be rapidly elicited to participate in the early stages of an immune response following activation through the secretion of IFN-γ or IL-17, due to the defined effector function they have acquired by the time their maturation in the thymus is complete through the described developmental pre-commitment, and unlike αβ CD4$^+$ T cells, that mostly leave the thymus as a 'naïve' cell population that further differentiates upon activation in the periphery into distinct CD4$^+$ Th cell subsets[12,15,31].

1.2.2. IFN-γ- and IL-17-producing γδ T cells

The proinflammatory cytokines IFN-γ and IL-17 are critical mediators of T cell responses against intracellular bacteria and viruses, or extracellular bacteria and fungi, respectively, and although they can derive from several cellular sources, γδ T cells are often major providers of both cytokines at early stages of immune responses[12,13].

While IFN-γ producing γδ T cells play protective roles in viral, parasitic and intracellular bacterial infections, such as *Listeria monocytogenes*[32,33], IL-17 producing γδ T cells contribute to protective immune responses to extracellular pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*, among other infections[13]. However, in addition to such protective roles, IFN-γ$^+$ and IL-17$^+$ γδ T cells can also elicit tissue damage and play a role in autoimmune diseases, such as multiple sclerosis or psoriasis[13,34], which overall illustrate the importance of understanding the mechanisms behind γδ T cell effector function, associated with its developmental pre-programming in the thymus.

1.3. microRNAs

There has been growing evidence that microRNAs (miRNAs), small (~22 nucleotides) single-stranded noncoding RNAs, are an integral part of gene expression networks, including T cell development and differentiation, determining cell identity and function by posttranscriptional repression of a target mRNA[35]. In fact, miRNAs and transcriptions factors are thought to modulate one another during development, contributing to the establishment of stable cellular identities[36].

In mammals, miRNAs are generally transcribed by RNA polymerase II to form a long primary transcript with a local stem-loop structure known as pri-miRNA (Figure 1.2.). The maturation process is initiated by the RNaseIII protein Drosha and its essential cofactor DiGeorge syndrome critical region gene 8 (DGCR8), which process the pri-miRNA to form a pre-miRNA, a ~60 nucleotide stem-loop structure which is then exported from the nucleus by exportin 5 via the nuclear pore, to be further processed in the cytoplasm by a second RNaseIII enzyme, Dicer, to form a miRNA duplex. This duplex is composed by two mature miRNAs, one from the 5' strand and one from the 3' strand of the precursor; usually only one of them is biologically relevant – the "guide" strand –, being loaded into an RNA-induced silencing complex (RISC)[37,38]. miRISC is then guided by the miRNA to target and repress an mRNA recognized by base pairing of the miRNA 5' end 'seed' domain (nucleotides 2 to 7)

to usually a sequence in the 3' untranslated region (UTR) of the target mRNA[35,37,38].

miRNAs act mainly as gene repressors at the level of transcriptional inhibition, either through mRNA decay, direct mRNA cleavage or miRNA-mediated chromatin reorganization, although they can also act at the translational level, through repression of initiation and/or elongation, premature termination and nascent polypeptide degradation[39]. Due to the shortness of the "seed" length, more than half of all protein-coding genes in mammals are regulated by miRNAs and a single miRNA can target several mRNAs[38]. In addition, non-conserved sites also exist, implying that most protein-coding genes may be under the control of miRNAs[37].

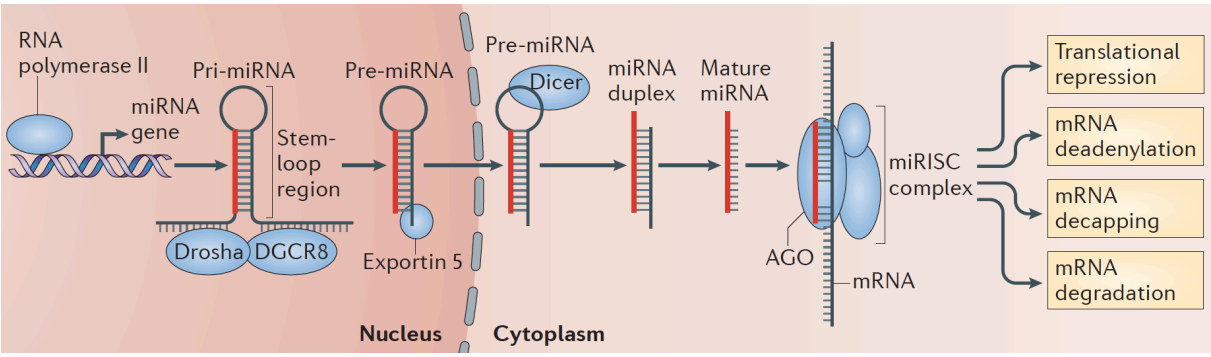

Figure 1.2. miRNA biosynthesis pathway. Schematic model of miRNA genomic transcription by RNA polymerase II, forming a pri-miRNA which is further processed by Drosha and DGCR8. The resulting pre-miRNA is then transported to the cytoplasm in association with exportin 5, where it is further processed by Dicer to form a miRNA duplex, from which a mature miRNA is incorporated into a RISC, an effector complex which either mediates translational repression, mRNA deadenylation, mRNA decapping or mRNA degradation. From Baumjohann *et al.*, *Nat. Rev. Immunol.* 13, 666-679 (2013)[35].

1.4. Control of T cell effector functions by miRNAs[1]

While Dicer-deficient embryos fail to develop[40], miRNA-deficient T cells generated by specific genetic inactivation of either Drosha, DGCR8 or Dicer exhibit reduced proliferation and survival after *in vitro* stimulation, as well as an increase in IFN-γ production, implying that miRNAs regulate T cell differentiation and effector functions[41–43].

So far, several miRNAs have been implied in the control of the IFN-γ and IL-17 production by the γδ T cells $CD4^+$ counterparts Th1 and Th17 cells, respectively, as well as in their differentiation[35,44]. For instance, miR-29 directly inhibits IFN-γ production by the Th1 cells through targeting T-bet and Eomesodermin (Eomes), two transcription factors that regulate IFN-γ expression, as well as IFN-γ itself[41,45], while miR-18b, miR-106a and miR-363 decrease Th17 cell differentiation and IL-17 production[46]. On the other hand, miR-326 promotes Th17 differentiation by targeting the TF Ets-1, a negative regulator of this process[47].

Besides regulating cytokine production, and partly through it, miRNA levels and their deregulation are associated with immune diseases, including multiple sclerosis[47,48]. In fact, aberrant miRNA expression in the immune system is sufficient to cause disease[35,49], highlighting the importance of the regulation of miRNA expression in the balance between health and disease and the necessity of their study.

Although several miRNAs have been shown to control the effector functions of several T cell subsets, little is known about how these post-transcriptional regulators act on γδ T cell development, functional differentiation and/or maintenance of effector function. miR-133 and miR-206 were the first miRNAs described to be co-regulated with the *il17a/f* locus in γδ T cells in terms of gene

[1] For a more extensive cover on the topic see our review 'Control of T cell effector functions by miRNAs': Inácio *et al.*, *Cancer Lett.* 427, 63-73 (2018)[44].

transcription. However, they did not impact cytokine production[50]. Only recently our group described such a crosstalk for γδ T cells by finding that miR-146a is selectively enriched in the IL-17-biased subset CD27$^-$ γδ T cells and restricts their IFN-γ production by targeting the Nod-like receptor *Nod1* mRNA and therefore regulates γδ T cell functional plasticity (Figure 1.3.)[51].

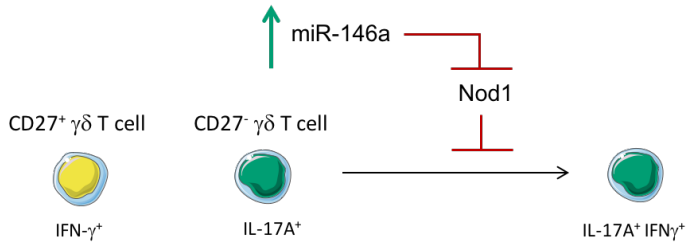

Figure 1.3. miR-146a controls functional plasticity in γδ T cells by targeting NOD1. miR-146a is thymically imprinted specifically in CD27$^-$ γδ T cells, which can differentiate into cells that produce simultaneously IL-17 and IFN-γ, acting as a post transcriptional brake to restrain their IFN-γ expression, through targeting *Nod1* mRNA.

2. Preliminary data

Following our discovery that miR-146a is mainly expressed in CD27$^-$ γδ T cells where it restrains their production of IFN-γ[51], we aimed at further dissecting miRNA-mediated regulation of γδ T cell differentiation by exploiting a novel layer of regulation independent of the use of surface markers, which do not allow isolation of pure populations of IL-17$^+$ or IFN-γ$^+$ γδ T cells. Thus, these pure γδ T cell populations were isolated from peripheral lymphoid organs of a double reporter IL-17-GFP:IFN-γ-YFP mouse strain and subjected to next generation sequencing analysis of both miRNA and mRNA repertoires which, for the first time, allowed the identification of miRNA and mRNA signatures directly associated with cytokine expression. Furthermore, following the rational that like miR-146a other overexpressed miRNAs in one functional population of γδ T cells could be supressing the production of the other major cytokine in that subset and therefore restraining γδ T cell plasticity, overexpressed miRNAs in one population were bioinformatically integrated with mRNA targets with a binding site on its 3' UTR that were known to be implicated in the other γδ T cell subset differentiation. This resulted in the establishment of two networks that allowed the prediction of miRNAs targeting key determinant mRNAs of the IL-17 and the IFN-γ programs of γδ T cells (Figure 2.1.A and B). From the identified miRNAs, 3 of them were selected for targeting genes in the IFN-γ program – miR-322-5p, miR-326-3p and miR-450b-3p – while 6 miRNAs were associated with the IL-17 program – miR-7a-5p, miR-92a-1-5p, miR-128-3p, miR-139-5p, miR-677-3p and miR-1949. These will be subject to a broader functional characterization on the identity and differentiation of effector γδ T cell subsets, which is the basis of the objectives for this MSc thesis (see bellow *3. Objectives*).

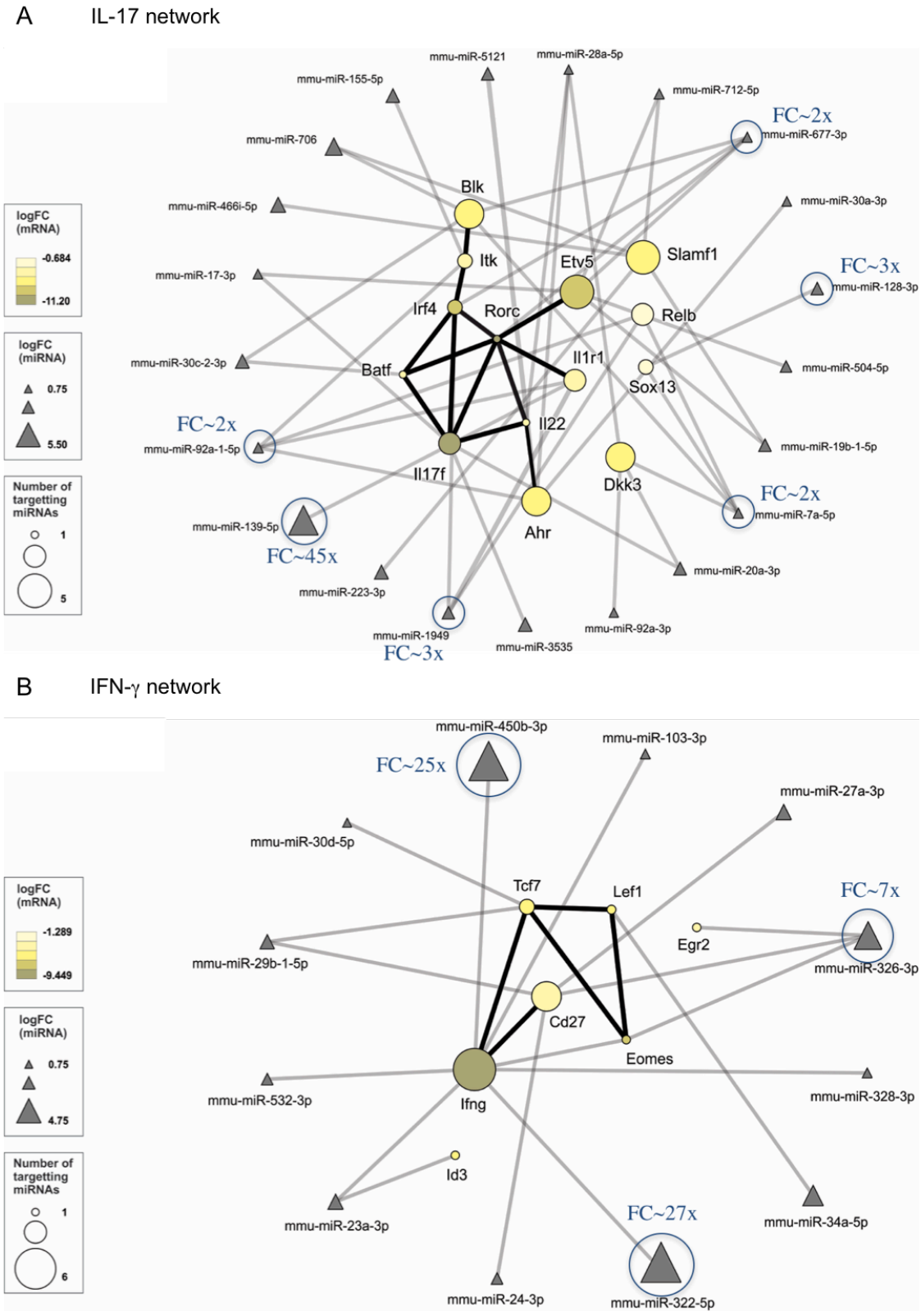

Figure 2.1. miRNAs predicted to target key determinant mRNAs of the IL-17 and IFN-γ gene expression networks of γδ T cells. miRNAs differentially expressed in IFN-γ^+ or IL-17^+ γδ T cells were bioinformatically integrated with mRNAs with a 3' UTR binding site described to be key determinants of the expression of the opposite cytokine by γδ T cells on the basis of miRNA-mRNA bioinformatic targeting prediction to obtain miRNAs predicted to target the IL-17 (**A**) or IFN-γ (**B**) gene expression network, respectively. Candidate miRNAs (identified in the figure) were selected for further functional characterization based on the level of its differential expression and the relevance of its predicted target(s) for the expression of the respective cytokine, as well as the number of targets.

3. Objectives

From the next generation sequencing analysis of the miRNA and mRNA repertoires of pure IL-17$^+$ and IFN-γ$^+$ γδ T cell populations isolated from peripheral lymphoid organs of a double reporter IL-17-GFP:IFN-γ-YFP mouse strain, miR-322-5p, miR-326-3p and miR-450b-3p were predicted to target key essential mRNAs of the IFN-γ gene expression program, while miR-7a-5p, miR-92a-1-5p, miR-128-3p, miR-139-5p, miR-677-3p and miR-1949 were predicted to target some determinant key genes of the IL-17 program.

While γδ T cell subsets can be functionally pre-programmed in the thymus to secrete IL-17 or IFN-γ, the set of events leading to their functional differentiation remains mostly unknown or controversial. Since miRNAs regulate temporal modifications in gene expression associated with cell fate progression and differentiation throughout development[36] and our group has previously found miR-146 to be thymically imprinted in CD27$^-$ γδ T cells and further maintained in the periphery[51], we set as our objective to approach the role of the above mentioned candidate miRNAs in the functional development and pre-programming of γδ T cells, comprising four main sub-objectives:

1. Validate the peripheral differential expression pattern of candidate miRNAs in foetal γδ T cell subsets;
2. Clone the selected miRNAs stem loops into a retroviral vector to overexpress its mature form;
3. Establish a system that allows for the modulation of miRNA expression in developing γδ T cells;
4. Screen for candidate miRNAs that functionally impact IL-17 or IFN-γ production during γδ T cell development.

4. Results

4.1. Expression levels of miRNAs in IFN-γ and IL-17 foetal thymic γδ T cells

To address the role of miRNAs in the development of γδ T cells pre-committed to the secretion of IL-17A or IFN-γ, we started by validating in foetal γδ T cell subsets the expression levels of the candidate miRNAs differentially expressed on adult peripheral γδ T cell subsets. To do so, we took advantage of the recently described strategy to segregate both functional development pathways through the use of the extracellular makers CD24, CD44 and CD45RB[24] (Figure 1.1.) and measured the *ex vivo* miRNA expression of highly purified IL-17 committed CD24$^-$CD44^{high}CD45RB$^-$ and IFN-γ committed CD24$^-$CD45RB$^+$ γδ T cells isolated from E17 thymic lobes (Figure 4.1.A) by quantitative reverse transcription polymerase chain reaction (RT-qPCR). All the candidate miRNAs revealed the same pattern of differential expression in the foetal thymic γδ T cell populations as in the corresponding adult peripheral γδ T cell subsets (Figure 4.1.B), with miRNAs predicted to target the IL-17 expression program overexpressed in the IL-17 pre-committed cells and vice-versa.

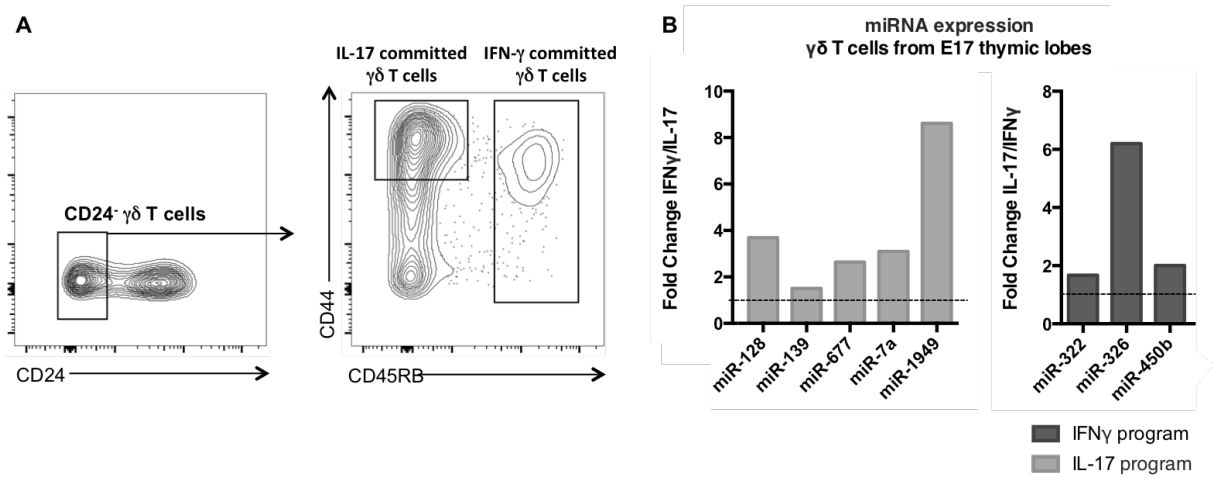

Figure 4.1. Candidate miRNA expression in foetal thymic γδ T cells. (**A**) Sorting strategy and (**B**) RT-qPCR expression analysis of the candidate miRNAs of the IL-17 program – miR-128, miR-139, miR-677, miR-7a and miR-1949 – and the IFN-γ program – miR-322, miR-326 and miR-450b – in the IL-17 pre-committed population $CD24^{-}CD44^{high}CD45RB^{-}$ and in the IFN-γ pre-committed population $CD24^{-}CD45RB^{+}$ γδ T cells from E17 thymic lobes; results were normalized to miR-423-3p (reference small RNA) and are presented relative to the opposite population. Data are representative of three technical replicates.

4.2. Gain of function studies of candidate miRNAs during γδ T cell development

Thinking of a functional approach to overexpress and concomitantly study the function of a miRNA in γδ T cell development, we cloned the native stem loops of each candidate miRNA with the adjacent flanking regions into a retroviral vector that would allow its overexpression through the normal miRNA-processing machinery of the cell, simultaneously encoding for green fluorescent protein (GFP). Viral particles were assembled in HEK 293T TAT cells through transfection of the viral plasmids and concentrated through high-speed centrifugation to produce a stock. This viral stock was tested in 3T3 cells for GFP expression through flow cytometry (Figure 4.2.A), in order to confirm transduction efficiency, and for miRNA expression (Figure 4.2.B), to confirm the success of mature miRNA overexpression by measuring their levels by RT-qPCR following RNA extraction and complementary DNA (cDNA) synthesis. Using between 1 and 10 µL of retrovirus (RV)-stock to transduce 3T3 cells, we managed to get more than 40% of GFP^{+} cells for all RV-miRNAs, detecting the correspondent miRNA overexpression in the pool of transduced and non-transduced cells (Figure 4.2.B), except for miR-677, that needs further validation.

With the miRNA overexpression tools available, we next needed a system that allowed retroviral transduction of developing γδ T cells. Although foetal thymic organ cultures (FTOCs) have been extensively used to study T cell development, we opted for Delta-like 1-expressing OP9 cell (OP9-DL1) co-cultures with hematopoietic progenitor cells due to its practical handling[52]. The OP9 stromal cell line was derived from the bone marrow of a macrophage colony-stimulating factor (MCSF) deficient mouse strain (OP/OP mice), limiting the clonal expansion of myeloid cells, and were further retrovirally transduced to express the Notch ligand delta-like 1, generating OP9-DL1, which are able to induce a normal programme of T-cell differentiation supressing that of B-cell lymphopoiesis, as opposed to the OP9 cell line[53]. As a source of lymphoid progenitors, we chose the E14-E15 foetal liver (Figure 4.3.A), as at this developmental stage this organ is still expected to contain progenitor cells from the first wave of cells colonizing the thymus, which are T-cell biased and give rise to foetal IL-17^{+} γδ T cells[54] (Table 1.1.). Still, by choosing the foetal liver instead of the thymus, we avoid to start the co-cultures with a contamination of lineage committed γδ T cells. To enrich the single cell

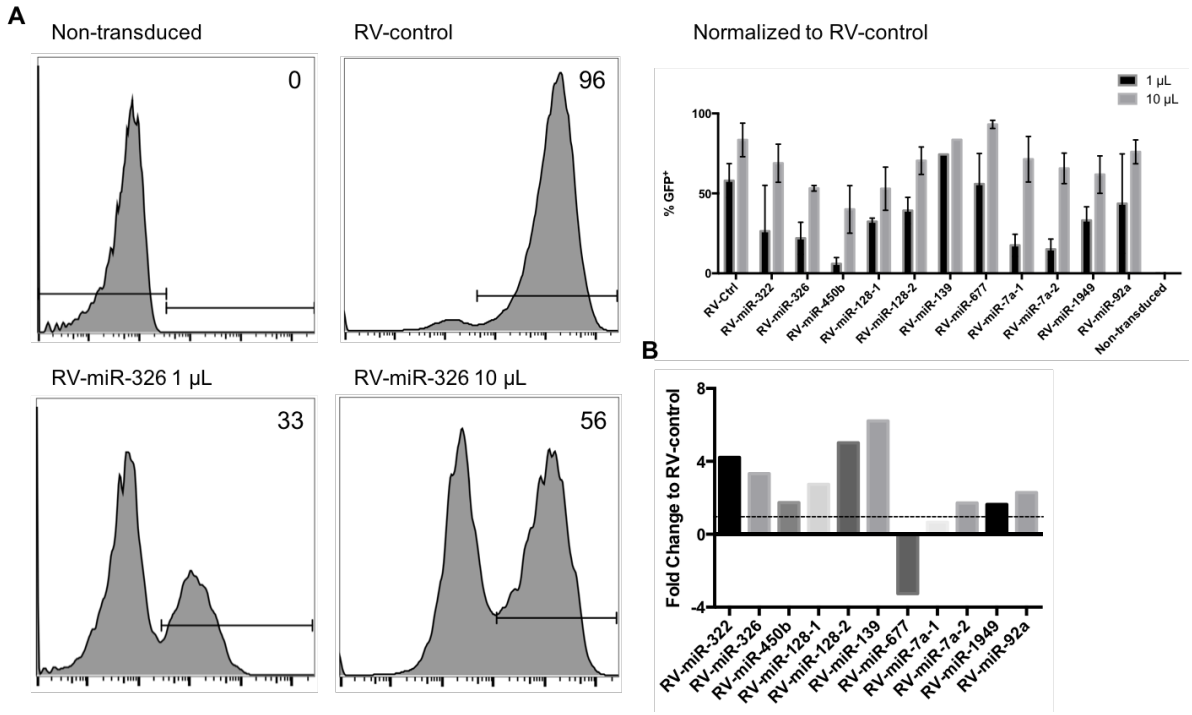

Figure 4.2. Production of miRNA-overexpressing retrovirus. (**A**) Flow cytometry analysis of GFP expression in retrovirally transduced 3T3 cells. (**B**) RT-qPCR analysis of the candidate miRNAs; results were normalized to the reference small RNA miR-423-3p and are presented relative to RV-control samples. Data are representative of several harvests from one or more batches of retroviral production.

suspension obtained from foetal livers with progenitor cells we performed a simple depletion of TER-119$^+$ cells by magnetic-activated cell sorting (MACS), which are reported to constitute 80-90% of E14 total foetal liver cells, accounting for erythroid cells at several stages of differentiation, from early proerythroblast to mature erythrocytes[55]. This fast and practical enrichment step allows the isolation of TER-119$^-$ cells with a purity of more than 99% a yield of around 90% (Figure 4.3.B). Foetal liver cells were then co-cultured with irradiated OP9-DL1 cells in medium containing Flt-3L and IL-7 (Figure 4.3.A), a combination of cytokines that has been shown to lead to a pronounced expression of TCRγδ after 16 days of co-culture of bone marrow progenitors with OP9-DL1 cells[56].

As we obtained very low frequencies of live co-culture derived lymphocytes after stimulating them with the general stimulus phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4h in the presence of the Golgi inhibitor brefeldin A in order to detect intracellular cytokine production by flow cytometry (Figure 4.3.C), we first analysed γδ T cell subsets according to extracellular markers associated with cytokine production, such as CD27, CD24, CD44, CD45RB, CD122 and NK1.1 (data not shown). However, we then decided to optimize this step in order to obtain live cells that allowed us to directly assess cytokine production by decreasing the time of incubation to 3h as well as the concentration of the stimuli (Figure 4.3.D). Although we were now able to get live cells, we could not detect a production of IL-17 or IFN-γ by γδ T cells developed solely with the cytokines Flt-3L and IL-7 (Figure 4.3.E), prompting us to test 69 combinations of extra cytokines, added at different time points of culture between days 0 and 10 (see Table 8.1 in *8. Annex*). Although we could never get more than 11% of IL-17$^+$ γδ T cells, we did obtain several conditions that allowed for the development of IFN-γ$^+$ γδ T cells in a frequency up to 90%. However, there was a general compromise between frequency and number of events (data not shown).

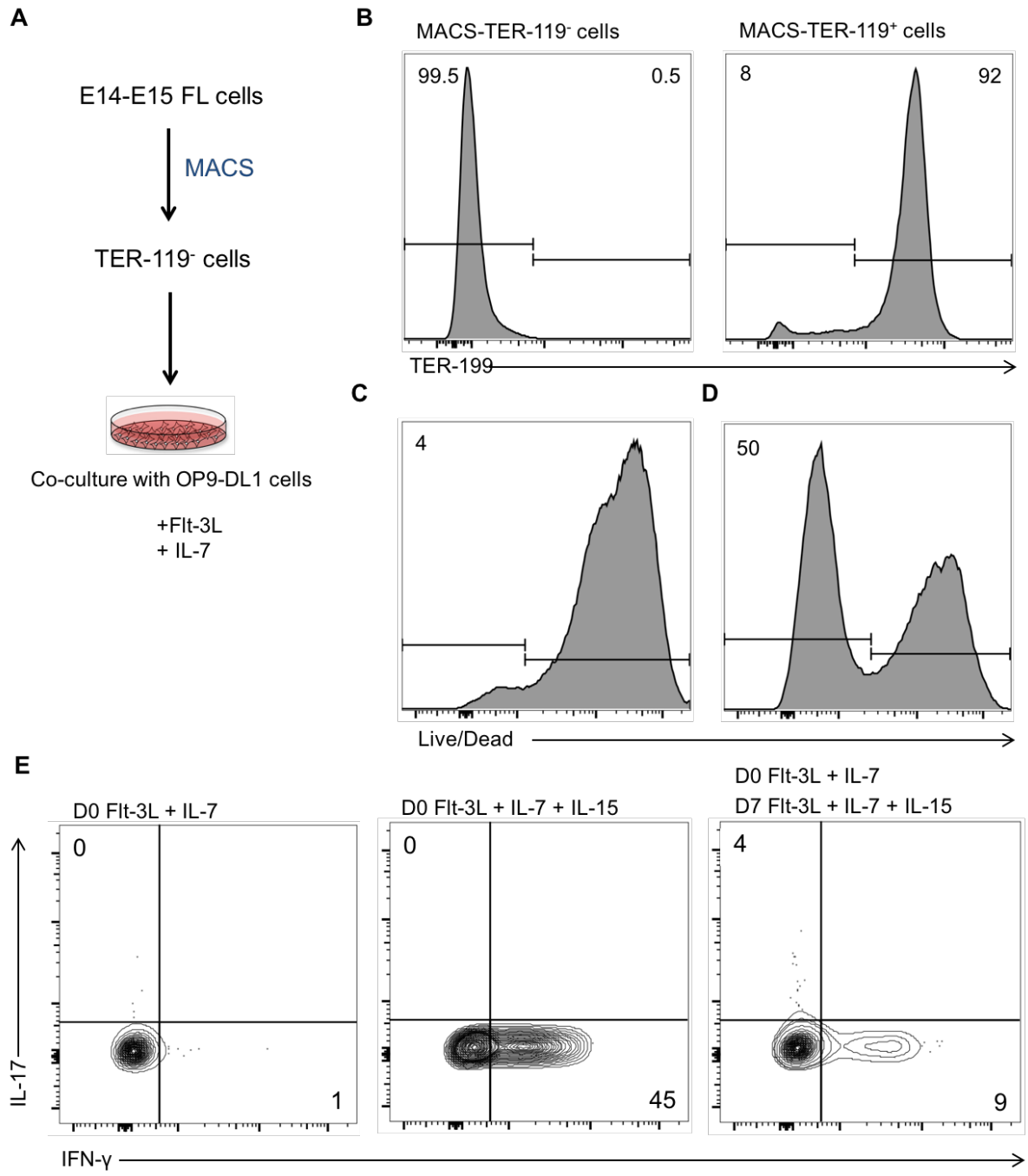

Figure 4.3. Optimization of an OP9-DL1 co-culture system to functionally modulate miRNA expression during γδ T cell development. (**A**) Workflow of the OP9-DL1 co-cultures with TER-119$^-$ foetal liver (FL) cells. (**B**) Flow cytometry analysis of TER-119 expression in MACS-TER-119$^-$ and TER-119$^+$ foetal liver single cell suspensions. (**C**) Flow cytometry analysis of live foetal liver TER-119$^-$ cells co-cultured with OP9-DL1 cells for 14 days and stimulated before analysis for 4 hours with PMA 100 ng/mL, ionomycin 4 µg/mL and brefeldin A 10 µg/mL and (**D**) for 3 hours with PMA 25 ng/mL, 1 µg/mL and brefeldin A 1 µg/mL. Frequency of live cells is indicated. (**E**) Flow cytometry analysis for intracellular IL-17 and IFN-γ production at day 10 of co-culture of TER-119$^-$ foetal liver cells with OP9-DL1 cells incubated with the indicated cytokine cocktails.

Since we failed to obtain IL-17$^+$ γδ T cells in a number and frequency high enough to allow for its modulation, we decided to focus on a condition that allowed for a good frequency of IFN-γ$^+$ γδ T cells to functionally modulate the miRNAs that had been predicted to target the IFN-γ program, namely miR-322, miR-326 and miR-450b. Since we wanted to study the candidate miRNAs in γδ T cell development and γδ T cells are reported to arise at the late DN2 to DN3 stage, we decided to transduce the co-cultured cells at day 4, since at this time point only 25% of the developing lymphocytes have

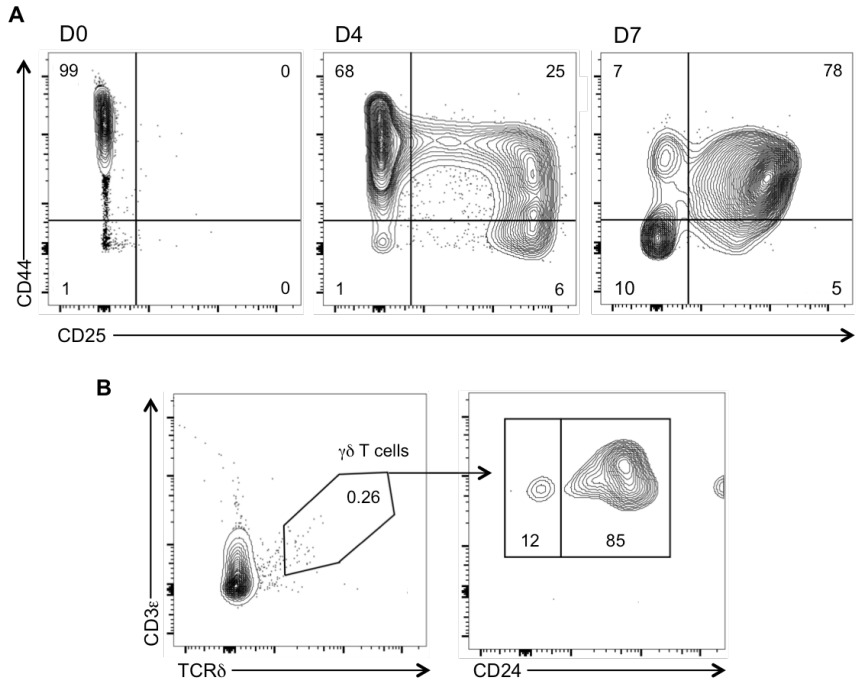

Figure 4.4. Assessment of T cell development in the OP9-DL1 co-cultures. (**A**) Flow cytometry analysis of lymphocyte DN stage at days 0, 4 and 7 and (**B**) of γδ T cells at day 4 of E15 TER-119$^-$ foetal liver cells co-cultures with OP9-DL1 cells in the presence of Flt-3L and IL-7. DN lymphocytes were gated on live CD45$^+$CD4$^-$CD8$^-$TCRδ$^-$ and γδ T cells on live CD45$^+$CD3ε$^+$TCRδ$^+$ cells.

passed from DN1 to DN2 stage, as opposed to 78% at day 7 (Figure 4.4.A), and γδ T cells were present in a very low frequency, and mostly immature as assessed by their positive expression of CD24 (Figure 4.4.B). With transduction set at day 4, we opted for a condition that allowed the analysis of transduced IFN-γ· γδ T cells at day 7, minimizing the period between miRNA overexpression and cytokine analysis. This condition consisted on the addition of IL-12 and IL-15 to the co-culture medium at day 4 after transduction (Table 8.1., Figure 4.5.A).

While none of the miRNAs seemed to impact on the general development of γδ T cells despite a tendency to promote γδ T cell lineage commitment (Figure 4.5.B), the retroviral transduction of the native stem loops of miR-326 and miR-450b in the progenitor cells led to a significant decrease in the frequency of IFN-γ$^+$ γδ T cells compared with a control vector alone, while that of miR-322 did not change it (Figure 4.5.C). To further validate these results, we confirmed the overexpression of the mature miRNAs in the co-cultures by RT-qPCR. While miR-326 and miR-450b were markedly overexpressed in the pool of the cells transduced with the respective RV-miRNA, miR-322 levels were only slightly overexpressed (Figure 4.5.D). These *in vitro* data suggest that both miR-326 and miR-450b are able to inhibit IFN-γ expression during γδ T cell development, supporting our initial hypothesis of their capacity to target the IFN-γ expression program in γδ T cells.

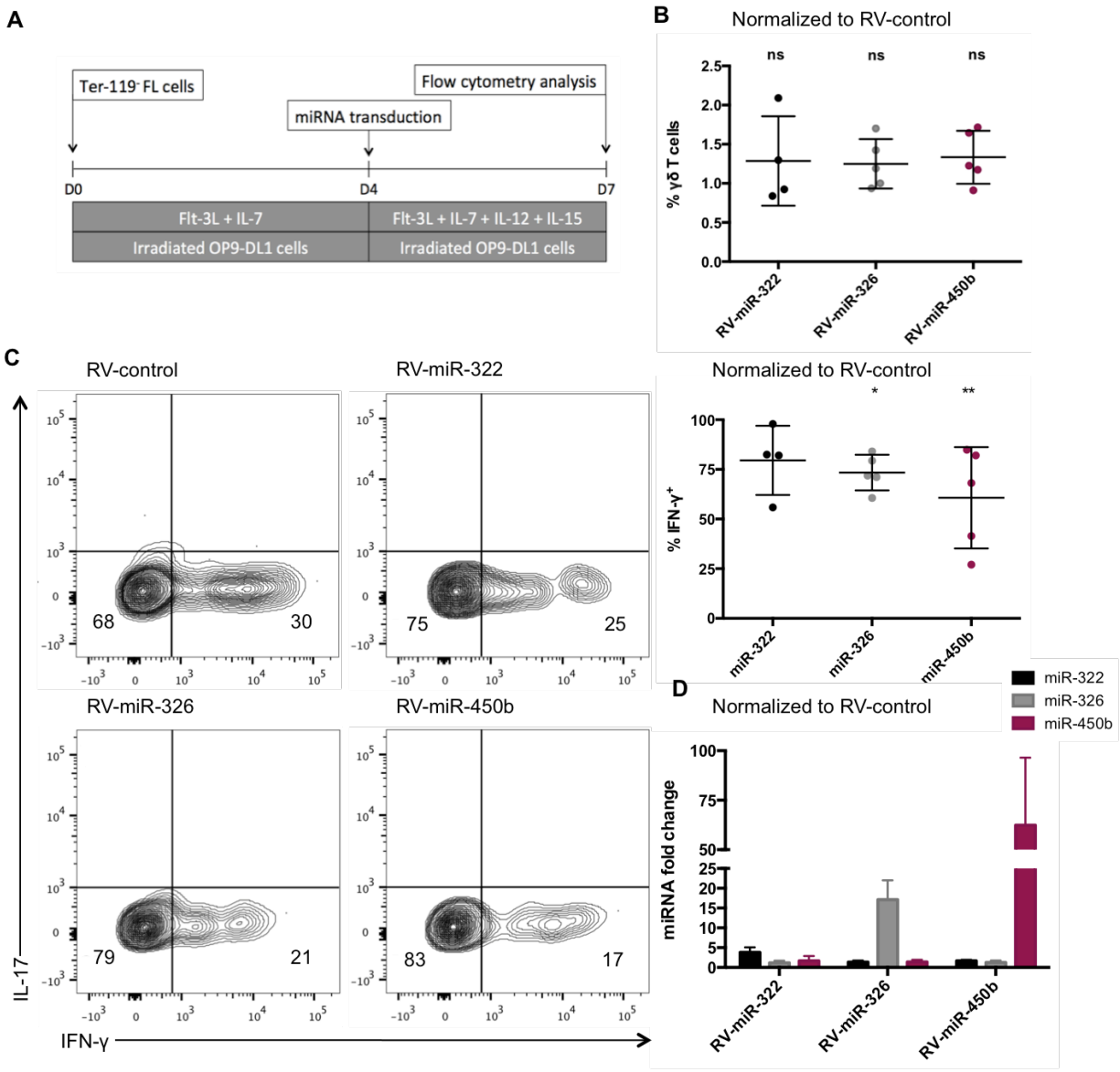

Figure 4.5. miR-326 and miR-450b inhibit IFN-γ production in developing γδ T cells. (**A**) Workflow of miRNA viral overexpression. (**B**) Frequency of γδ T cells and (**C**) flow cytometry analysis of intracellular IFN-γ and IL-17 production by γδ T cells and frequency of IFN-γ^+ γδ T cells in retrovirally transduced developing E15 foetal liver TER-119^- progenitor cells in co-culture with OP9-DL1 cells with either RV-control, RV-miR-322, RV-miR-326 or RV-miR-450b; gated on live $GFP^+CD45^+CD3\varepsilon^+\gamma\delta^+$ cells. (**D**) RT-qPCR analysis of miR-322, miR-326 and miR-450b expression in total cells of (B); results were normalized to the reference small RNA miR-423-3p and are presented relative to RV-control samples. Data are representative of four to five (B and C) and four (D) independent experiments with three technical replicates each. **P*<0.05 and ***P*<0.01.

4.3. Regulation of candidate miRNA expression on developing γδ T cells

In order to better understand the changes in gene expression of miR-326 and miR-450b in developing γδ T cells, we measured once again the miRNA expression levels of the IFN-γ program miRNA candidates this time in three thymic populations of γδ T cells: the uncommitted CD24$^-$CD44$^-$ CD45RB$^-$, the IL-17 pre-committed CD24$^-$CD44$^+$CD45RB$^-$ and the IFN-γ pre-committed CD24$^-$ CD45RB$^+$ γδ T cells isolated from E17 thymic lobes by RT-qPCR. While miR-322 expression remained the same as cells entered the IL-17 pathway, miR-326 and miR-450b levels increased more than 2 fold (Figure 4.5.A). On the other hand, the levels of miR-326 further decreased in the γδ T cells that entered the IFN-γ pathway. These results suggest not only a functional role for miR-326 and miR-450b in γδ T cell development, but also that differentially expressed miRNAs in peripheral γδ T cells might be under tight regulation already during foetal thymic γδ T cells, further validating γδ T cell pre-commitment. In fact, we performed the same analysis for the IL-17-targeting candidate miRNAs, and while all miRNAs were downregulated in the IL-17 pre-committed population, miR-7a, miR-92a, miR-677 and miR-1949 were simultaneously upregulated in the IFN-γ-biased population (Figure 4.5.B.), supporting the relevance of the thymic γδ T cell pre-programming in the establishment of their peripheral miRNA signatures.

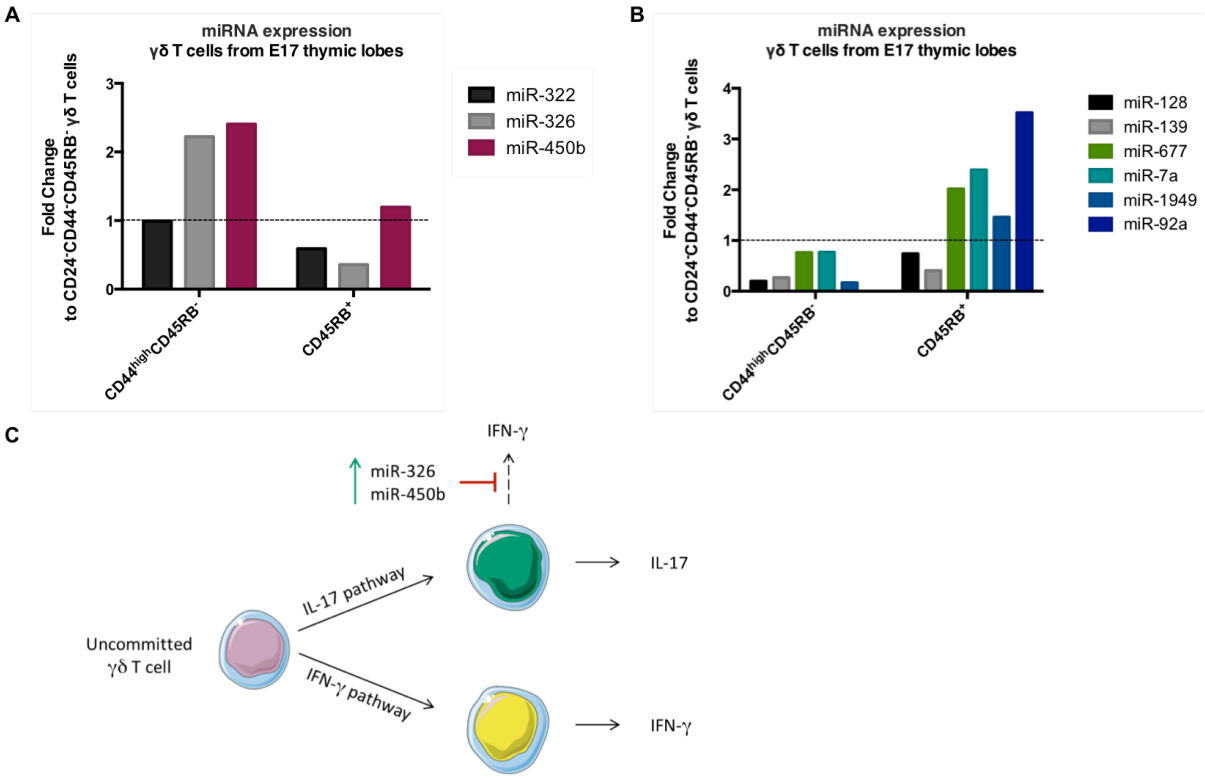

Figure 4.6. Candidate miRNA expression throughout thymic γδ T cell lineage commitment. (**A**) RT-qPCR analysis of miRNA expression of the IFN-γ program candidates miR-322, miR-326 and miR-450b and (**B**) of the IL-17 program candidates miR-128, miR-139, miR-677, miR-7a, miR-1949 and miR-92a in the IL-17 prone population CD24$^-$ CD44^{high}CD45RB$^-$ and in the IFN-γ prone population CD24$^-$CD45RB$^+$ γδ T cells from E17 thymic lobes relative to that of their mature uncommitted population counterpart CD24$^-$CD44$^-$CD45RB$^-$; results were normalized to the reference small RNA miR-423-3p. (**C**) A novel model for miRNA regulation of γδ T cell thymic pre-commitment: miR-326 and miR-450b levels specifically increase in thymic developing γδ T cells that enter the IL-17 pathway, restraining their capability to co-express IFN-γ.

Overall, the specific increase in miR-326 and miR-450b levels as γδ T cells transit from an uncommitted to an IL-17 committed subset together with their capability of supressing IFN-γ

production *in vitro* in developing γδ T cells suggests an epigenetic mechanism of regulation, according to which both miR-326 and miR-450b are thymically imprinted in γδ T cells that enter the IL-17 pathway to restrain their IFN-γ expression, possibly regulating γδ T cell plasticity (Figure 4.5.B).

5. Discussion

miRNAs are a large family of endogenous noncoding RNAs responsible for a fundamental layer of post-transcriptional regulation of gene expression[36,37]. In the present study our group performed an *ex vivo* next generation sequencing analysis of both miRNA and mRNA repertoires of pure IL-17$^+$ and IFN-γ$^+$ γδ T cells, from which, an integrated bioinformatic approach predicted miR-7a, miR-92a, miR-128, miR-139, miR-677 and miR-1949 to target the IL-17 gene expression network and miR-322, miR-326 and miR-450b to target the IFN-γ expression program. Overall, the aforementioned miRNAs followed the same expression pattern in the corresponding foetal γδ T cells developing in the thymus. Due to technical limitations with γδ T cell development *in vitro* models, we could only test the IFN-γ program-targeting candidate miRNAs for a functional effect on cytokine expression by developing γδ T cells. miR-326 and miR-450b were found to regulate γδ T cell development by inhibiting IFN-γ production. Furthermore, the expression of both miRNAs specifically increased in γδ T cells as they commit to the IL-17 functional subset in the thymus, and not in the γδ T cells biased towards IFN-γ production. This supports the concept of thymic imprinting in IL-17$^+$ γδ T cells to restrain their capability to co-express IFN-γ.

We found that miR-322, miR-326 and miR-450b peripheral γδ T cell signatures were set in the corresponding foetal thymic γδ T cell population. This is in accordance with our previous findings showing that miR-146a was differentially expressed during γδ T cell development in the IL-17 biased population CD27$^-$ γδ T cells[51]. Importantly, our results on miR-326 and miR-450b add to our knowledge of the epigenetic mechanisms of regulation of γδ T cell effector function. In fact, it was first found by our group that while CD27$^+$ γδ T cells are committed to the expression of *Ifng* but not *Il17*, CD27$^-$ γδ T cells have permissive chromatin configurations at loci encoding both cytokines and the respective transcription factors, being able to differentiate into cells that express both cytokines but only under certain inflammatory circumstances, such as the tumour microenvironment[57]. Our current model may clarify a key mechanism by which under homeostasis these cells are able to maintain their identity as IL-17 producers.

miR-450b is predicted to target directly the 3' UTR of the *ifng* mRNA and miR-326 is predicted to target the IFN-γ program of γδ T cells through the targeting of three of its components: CD27, Egr2 and Eomes. Regarding CD27, the expression of this member of the tumour necrosis family on the cell surface of γδ T cells is associated with IFN-γ secretion and it participates in the regulation of their differentiation in part by inducing genes associated with IFN-γ production[31]. As for the transcription factors, while Egr2 is associated with γδ T cells IFN-γ pathway, promoting Id3 expression and suppressing the IL-17 pathway, Eomes is an auxiliary transcription factor that contributes to the differentiation of the IFN-γ-producing Th1 cells but which is dispensable in γδ T cells[58,59]. Considering the predicted targets of both miR-450b and miR-326, it is not surprising that miR-450b seems to have a greater impact on IFN-γ inhibition, given its direct targeting of the key cytokine mRNA instead of its potential regulators.

While there are still no reports about miR-322 and miR-450b in cells of the immune system, miR-326 is a Th17 cell-associated miRNA, promoting Th17 differentiation by targeting Ets-1, a negative regulator of Th17 differentiation[47]. In fact, its expression is correlated with disease severity both in multiple sclerosis patients and in the respective mouse model experimental autoimmune disease

(EAE), with its *in vivo* silencing leading to fewer Th17 cells and mild EAE and its overexpression leading to more Th17 cells and severe EAE[47]. However, no differences in IL-17 expression were reported in γδ T cells of mice infected with a control lentivirus when compared with mice overexpressing either miR-326 lentivirus or a miR-326 knockdown-sponge[47]. To our knowledge, Ets-1 has not been implied in the differentiation of γδ T cell effector subsets, and its deficiency does not change the overall γδ T cell number[60]. In our study miR-326 was also overexpressed in γδ17 T cells, and although we hypothesised it to downregulate IFN-γ through the key predicted target mRNAs of IFN-γ pathway, it could as well be the case that it functions to increase the differentiation and pathogenicity of γδ17 T cells in a similar way to Th17 cells. Either way, miR-326 promoting IL-17$^+$ CD4$^+$ T cells but not the IL-17$^+$ γδ counterparts serves to illustrate both the difficulty of translating the knowledge of the miRNA control of the effector function from one cell type to the other and the need to discover miRNAs with the potential to modulate the effector γδ T cell function.

Although γδ17 T cells arise in FTOCs[24], we did not use this T cell development system due to its low cell yields and varying seeding efficiency adding to a complicate manipulation of the progenitor cells during differentiation[61]. It would be difficult not only to transduce the cells but also to get a reasonable final number of transduced γδ T cells to analyse by flow cytometry, implying the use of a large number of starting material to perform the whole miRNA screening. While *Rag2*$^{-/-}$ DN3 cells transduced with the γδ TCR KN6 (Vγ4Vδ5) co-cultured on mouse embryonic fibroblasts (MEFs) expressing the Notch ligand Dll4 give rise to γδ17 T cells in the presence of IL-1β, IL-21 and IL-23[25], and this cytokine combination also drives γδ17 T cell differentiation in infection settings[62–64], we were not able to reproduce these results with our progenitor cells in the OP9-DL1 system. Although we managed to get a maximum of 11% of IL-17$^+$ γδ T cells, we did not consider the percentage and final number of events robust enough for observing its modulation in the presence of a given miRNA. Indeed, in spite of the extensive use of this system to approach T cell development, so far no one has reported the expansion of γδ17 cells. It is possible that this is a caveat of this bidimensional model that either does not recapitulate all the molecular cues to which thymocytes precursors are naturally exposed in the thymic microenvironment[52], or that lacks the proper niche for γδ17 T cell development. In fact, weak TCR signalling has been proposed to be optimal for γδ17 T cell development, while strong TCR-ligand interactions would rather drive γδIFN-γ development and correlate with *Tbx21* upregulation[22–25]. Thus it is possible that OP9-DL1 cells express TCR ligands that drive preferentially IFN-γ producers. Although this is a difficult issue to address, since TCRγδ ligands are mostly unknown[12], the γδ TCR $V_\gamma4V_\delta5$, which is associated with γδ17, recognizes the non-classical MHC1b molecules T10 and T22, expressed by OP9 cells and the latter of which is a strong ligand for that TCR[25] and could prohibit the pre-commitment to the IL-17 pathway by developing γδ T cells. In fact, our lab has recently participated in a study that showed that strong TCR signalling impedes γδ17 T cell development[24].

Nevertheless, the optimization of the OP9-DL1 co-cultures model and the trials with several cytokines cocktails added at different time points of culture together with different time point analysis generated a large amount of data which may prove useful to address different questions on γδ T cell development or even T cell development on a broader scale.

It has been shown that some miRNAs found in immune cells, in addition to being able to regulate cytokine production, are themselves modulated in response to inflammatory stimuli and proinflammatory cytokines[65,66], in a dual cross-talk between the both types of regulating-molecules[48]. We were interested in gaining insight into the regulation of miR-322, miR-326 and miR-450b. In this line of thought, we gathered some preliminary data on the upstream regulation of miRNA expression taking advantage of our OP9-DL1 co-cultures system to approach γδ T cell development. We

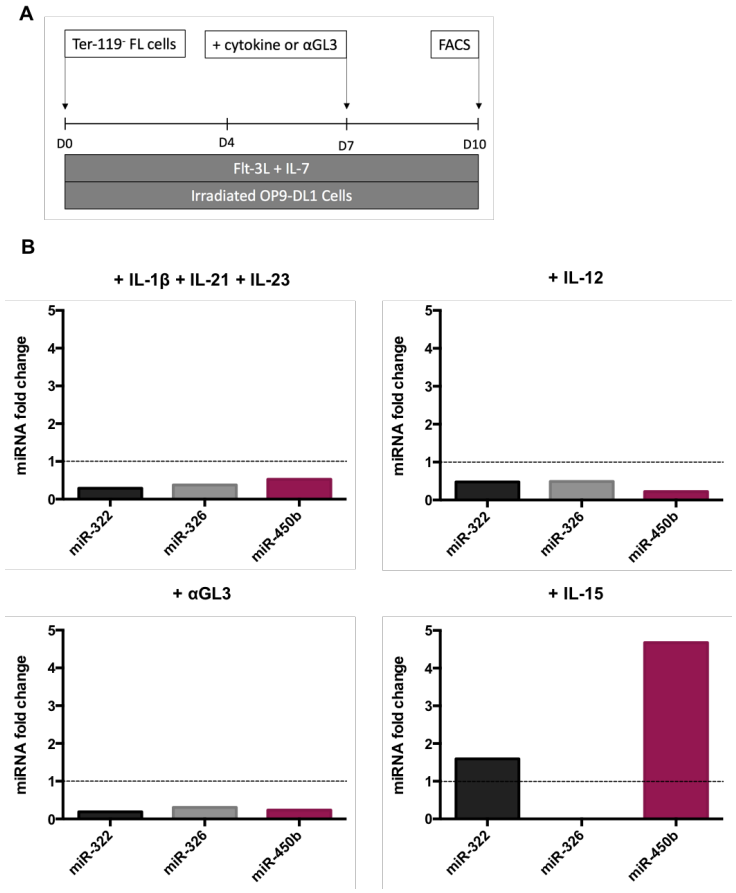

Figure 5.1. Upstream regulation of IFN-γ expression program candidate miRNAs. (**A**) Workflow of the preliminary study of the upstream regulation of miR-322, miR-326 and miR-450b: TER-119- cells from E15 foetal livers were added to OP9-DL1 co-cultures supplemented with Flt-3L + IL-7; on day 7 anti-GL3, IL-12, IL-15 or IL-1β + IL-21 + IL-23 were added, and γδ T cells were sorted on day 10. (**B**) RT-qPCR analysis of the γδ T cells from (A); miRNA levels were normalized to reference miR-423-3p and are presented as relative to the control condition with only Flt-3L + IL-7.

measured the relative expression levels of the respective mature miRNAs by RT-qPCR in highly purified γδ T cells developed in OP9-DL1 co-cultures to which different stimuli were added 3 days prior to analysis (Figure 5.1.A). Preliminary results showed that compared to control, the levels of the three miRNAs decreased with either TCR stimulus (anti-GL3), IL-12 or the γδ17 polarising cytokine cocktail IL-1β + IL-21 + IL-23, all of which are inflammatory signals (Figure 5.1.B). miR-322 and miR-326 levels increased only in the condition to which the homeostatic cytokine IL-15 was added, but not those of miR-326, which was not detected.

Overall, while inflammatory stimuli seem to decrease miR-322, miR-326 and miR-450b levels, the homeostatic cytokine IL-15 seems to increase miR-322 and miR-450b levels but not miR-326 levels. In fact, both IL-12 and a strong TCR stimulus, such as the anti-GL3, would induce IFN-γ production[24,67]. Since miR-322, miR-326 and miR-450b are overexpressed in IL-17$^+$ in relation to IFN-γ$^+$ γδ T cells, a decrease in their levels would be expected with IFN-γ promoting stimuli. On the other side, IL-1β, IL-21 and IL-23 have been shown to differentiate γδ T cells into IL-17 producers[25], leading us to expect an increase in these miRNA levels. However, since we could not observe IL-17$^+$ γδ T cells in these cultures by flow cytometry (data not shown), it could as well be that our γδ T cells somehow did not receive their signalling but instead other cells derived from the TER-119$^-$ progenitor cells present in the co-culture did. These cells would, in turn, provide inflammatory cues that would bias γδ T cells towards IFN-γ producers, leading to a decrease in the miRNA levels. As for IL-15, while studies with IL-15Rα knockout mouse suggest that the α chain of its receptor

intrinsically constrains the development of γδ17 T cells, IL-15 knockout mouse do not display such a phenotype and it could be possible that another ligand drives that downstream effect independent of IL-15[68]. In fact, IL-15 has been shown to induce the *in vivo* expansion of γδ17 T cells[69]. In our system, IL-15 promoted miR-322 and miR-450b expression, a miRNA signature associated with γδ17 T cells, supporting the hypothesis that another ligand that not IL-15 induces the γδ17 T cell-restraining signalling by IL-15Rα. The fact that only the levels of these miRNAs increase but not those of miR-326 could be justified by the observation that the first ones are integrated in the same genomic cluster in the X chromosome and transcribed in the same primary transcript, therefore sharing the same promotor, which remains to be identified[70]. It should be noted that, although preliminary, these results indicate that further dissection of the regulation of these miRNA expression levels should be done in a simpler culture system of pure γδ T cells instead of co-cultures with multiple lymphoid cell subsets. In this system, Notch signalling should be modulated in order not to be confounded as an additional stimulus. A detailed bioinformatic approach should be done as well to find probable miRNA promotor regions and the respective regulators.

Future work will also have to address the functional effect of all the candidate miRNAs in IL-17 and IFN-γ production by peripheral γδ T cells. We are currently establishing several models in our lab which will be more suitable for the assessment of other broader potential roles of miRNAs on γδ T cells that we did not address here, like proliferation, cell death and metabolism. For the miRNAs found to indeed control γδ T cell effector function, a target screen should be performed in order to further dissect their mechanism of action, and the whole analysis should be validated in *in vivo* mouse models.

In sum, our data points towards miR-326 and miR-450b being specifically imprinted in developing γδ T cells that pre-commit to the IL-17 expression program to restrain their co-expression of IFN-γ. These insights into the mechanisms that control γδ T cell differentiation and function may be of clinical interest, since the manipulation of the levels of miRNAs that decrease IFN-γ or IL-17 production by γδ T cells may have therapeutic potential in pathologies in which these cells have been described to play a pathogenic role, like cancer or autoimmunity.

6. Materials and Methods

Mice: C57BL/6J mice were purchased from the Charles Rivers Laboratories. All mice were foetal (E14-E17). Embryos were obtained from timed pregnancies. 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.

Tissue processing and cell isolation: Cell suspensions were obtained from E14-E15 foetal livers or E17 thymic lobes. Single-cell suspensions were obtained after the organs had been gently teased apart in fluorescence-activated cells sorting (FACS)-buffer [phosphate-buffered saline (PBS) containing 2% heat-inactivated foetal calf serum (FCS)] and filtered through 70-μm cell strainers (BD Biosciences). For thymocyte cell suspensions, erythrocytes were osmotically lysed in red blood cell lysis buffer (BioLegend). Foetal liver cell suspensions were instead sorted by magnetic-associated cell sorting using LS columns (Myltenyi Biotech) to deplete TER-119$^+$ cells (eBioscience).

Cell Culture: HEK 293 TAT cells were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose with L-glutamine supplemented with 10% heat-inactivated FCS and 1%

penicillin and streptomycin. 3T3 cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin and streptomycin, 1% HEPES and 1% non-essential aminoacids (NEAA). OP9-DL1 cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin and streptomycin, 1% NEAA and 0.1% β-mercaptoethanol. All cell lines were passaged every 2-4 days and maintained below total confluence. All reagents were from Gibco.

Retroviral production: The retroviral constructs encoding either miR-322-5p, miR-326-3p, miR-450b-3p, miR-7a-5p, miR-92a-1-5p, miR-128-3p, miR-139-5p, miR-677-3p or miR-1949 were generated by inserting the respective native pre-miRNA sequences flanked by about 200 bp into a modified pMig.Ires-GFP retroviral vector (Addgene)[51] using HD In Fusion kit (Takara Bio USA). miRNA sequences were obtained by PCR amplification from genomic DNA from C57BL/6J mice. Retroviral particles were assembled in HEK 293T TAT cells transfected with the miRNA-containing vector or control vector alone together with the additional viral plasmids pCL-Eco and pCMV-VSV (both from Addgene), in the presence of HEK 293 TAT medium supplemented with Opti-MEM (Gibco) and X-tremeGENE 9 (Sigma-Aldrich). Supernatant-containing retroviral particles were collected between 48h-96h and concentrated through high speed centrifugation for 2h at 75600 g at 4 °C. To test the virus for GFP expression and miRNA overexpression, 3T3 cells were transduced in the presence of polybrene (4 μg/mL; Sigma-Aldrich) and subsequently analysed after 48 hours by flow cytometry or frozen in liquid nitrogen for later RNA extraction, cDNA synthesis and RT-qPCR.

OP9-DL1 co-cultures and miRNA retroviral overexpression: The OP9-DL1 cell line was provided by Dr. Daniel Pennington. Cells from foetal livers were seeded onto a semi-confluent monolayer of OP9-DL1 cells previously irradiated (1000 rad) cultured in 24-wells plate with 500 μL of OP9-DL1 medium per well supplemented with 5 ng/mL Flt-3L ligand (StemCell) and 1 ng/mL IL-7 (PeproTech). Cells were incubated for 7-10 days at 37 °C and 5% CO_2 and the OP9-DL1 monolayer and the respective medium with cytokines were replenished on days 4, 7 and 10 of co-culture, depending on its duration. For retroviral overexpression of miRNAs at day 4, cells from each well washed in PBS and filtered through a 40 μm cell strainer and transduced in the presence of polybrene (4 μg/mL; Sigma-Aldrich) for 90 min at 30 °C before being washed again and ressuspended in OP9-DL1 medium supplemented with IL-12 (5 ng/mL, PeproTech) and IL-15 (10 ng/mL, PeproTech). Cultured cells were subsequently analysed by flow cytometry at day 7, 10 or 13-14 or frozen in liquid nitrogen for later RNA extraction, cDNA synthesis and RT-qPCR. In other experiments anti-TCRδ antibody (GL3; 1 μg/mL; StemCell), IL-12 (5 ng/mL), IL-15 (10 ng/mL), IL-12 (5 ng/mL) and IL-15 (10 ng/mL) or IL-1β (5 ng/mL, PeproTech), IL-21 (20 ng/mL, PeproTech) and IL-23 (10 ng/mL, R&D Systems) were added to the cultures at day 7, and cultured cells were sorted at day 10 and frozen in liquid nitrogen. Cultures containing anti-TCRδ were rested overnight in fresh OP9-DL1 medium before analysis.

Flow cytometry and cell sorting: Anti-mouse fluorescently labelled monoclonal antibodies (purchased from BD Biosciences, eBioscience or BioLegend) were used against the following cell surface proteins and cytokines: CD3ε (145-2C11), CD24 (M1/69), CD44 (IM7), CD45 (30-F11), CD45RB (C363.16A), TCRδ (GL3), TCRβ (H57-597), IFN-γ (XMG1.2) and IL-17A (TC11.18H10.1). For cell surface staining cells were incubated at 4 °C with fluorochromes-conjugated antibodies diluted in FACS buffer in the presence of anti-CD16/CD32 (eBioscience) to block Fc receptors and of LIVE/DEAD (Life Technologies) for dead cell exclusion prior to cell sorting on a FACSAria (BD Biosciences) or flow cytometry analysis in a FACSFortessa (BD Biosciences). For flow cytometry analysis of cytokine analysis, cells were previously stimulated for 3 hours at 37 °C with PMA (25 ng/mL) and ionomycin (1 μg/mL) in the presence of brefeldin A (1 μg/mL) (all from

Sigma-Aldrich). After surface staining like described, cells were fixed 30 min at 4 °C with paraphormaldeyde (PFA) 4% (Sigma-Aldrich), permeabilized with Permeabilization Buffer (eBioscience) in the presence of anti-CD16/CD32 for 15 min, following which intracellular staining was performed by adding antibodies against cytokines in the indicated permeabilization and incubating for 1 hour at 4 ºC. Flow cytometry data were analysed using FlowJo software (TreeStar).

RNA isolation, complementary DNA synthesis and real-time quantitative PCR: RNA was isolated using miRNeasy Mini Kit (Qiagen). For miRNA, reverse transcription was performed with a Universal cDNA Synthesis kit II (Exiqon). cDNA of miRNA samples was quantified using the respective miRNA LNA primers (Exiqon) relatively to miR-423-3p using SYBR (Alfagene) on a ViiA7 cycler (Applied Biossystems).

Statistical analysis: Data are presented as mean ± s.d. The statistical significance of differences between populations was assessed using one-way analysis of variance (ANOVA). A difference was considered significant if $P \leq 0.05$.

7. References

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8. Annex

8.1. Supplementary table

Table 8.1. Optimization of the OP9-DL1 co-cultures system to study γδ T cell development. List of tested conditions in the OP9-DL1 co-cultures with TER-119$^-$ E14-E15 FL cells with respective frequencies of γδ T cells and their IL-17 and IFN-γ production analysed by flow cytometry. The orange background refers to the analysis day. In every mentioned day prior to the analysis one, co-culture wells were washed and filtered through a 45 µm cell-strainer to remove cell clumps and larger OP9-DL1 cells and seeded onto a new irradiated OP9-DL1 monolayer with renewed medium and cytokines. Data are representative of one to seven replicates.

D0	D4	D7	D10	D13-14	Analysis		
					%γδ T cells	%IFN-γ$^+$	%IL-17$^+$
+ IL-7	+ IL-7	+ IL-7			0,7	2,5	0,4
		+ IL-7	+ IL-7		6,4	1,7	2,5
			+ IL-7	+ IL-7	13	1,0	1,2
+ IL-7*	+ IL-7*	+ IL-7*			0,6	0,0	3,5
			+ IL-7*		6,3	1,3	0,2
+ IL-7	+ IL-7	+ IL-15	+ IL-15		6,6	3,9	10,7
			+ IL-15	+ IL-15	1,2	51,5	4,9
+ IL-7	+ IL-7	+ IL-7 + IL-12	+ IL-7 + IL-12		4,3	3,0	2,2
			+ IL-7 + IL-12	+ IL-7 + IL-12	2,0	0	0
+ IL-7	+ IL-7	+ IL-7 + IL-15	+ IL-7 + IL-15		6,4	6,9	2,0
			+ IL-7 + IL-15	+ IL-7 + IL-15	8,1	10,9	1,1
+ IL-7	+ IL-7	+ IL-12 + IL-15	+ IL-12 + IL-15		2,2	1,6	7,9
			+ IL-12 + IL-15	+ IL-12 + IL-15	0,0	0,0	0,0
+ IL-7	+ IL-7	+ IL-7 + IL-12 + IL-15	+ IL-7 + IL-12 + IL-15		4,7	6,8	2,1
			+ IL-7 + IL-12 + IL-15	+ IL-7 + IL-12 + IL-15	4,3	12,3	2,7
+ IL-7	+ IL-7	+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23		4,1	2,9	2,0
			+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23	7,2	3,1	0,6
+ IL-7	+ IL-7	+ IL-7 + IL-12 + IL-15 + IL-1β + IL-23	+ IL-7 + IL-12 + IL-15 + IL-1β + IL-23		4,2	15,7	5,9
			+ IL-7 + IL-12 + IL-15 + IL-1β + IL-23	+ IL-7 + IL-12 + IL-15 + IL-1β + IL-23	0	0	0
+ IL-7	+ IL-15	+ IL-15			3,3	14,1	0,8
		+ IL-15	+ IL-15		0,6	64,3	0,9
			+ IL-15	+ IL-15	0,5	90,0	1,0
+ IL-7	+ IL-7 + IL-15	+ IL-7 + IL-15			1,0	13,2	0,6
		+ IL-7 + IL-15	+ IL-7 + IL-15		4,2	15,4	0,7
			+ IL-7 + IL-15	+ IL-7 + IL-15	2,4	11,9	1,3
+ IL-7	+ IL-7 + αIFN-γ	+ IL-7 + αIFN-γ			0,4	0	0
		+ IL-7 + αIFN-γ	+ IL-7 + αIFN-γ		7,6	4,4	0,1
+ IL-7	+ IL-7 + αGL3	+ IL-7 + αGL3			1,1	0,0	4,2
		+ IL-7 + αGL3	+ IL-7 + αGL3		0,7	11,4	4,6
+ IL-7	+ IL-7 +IL-12 + IL-15	+ IL-7 +IL-12 + IL-15			1,2	14,2	0,9
		+ IL-7 +IL-12 + IL-15	+ IL-7 +IL-12 + IL-15		1,7	21,7	0,3
			+ IL-7 +IL-12 + IL-15	+ IL-7 +IL-12 + IL-15	0,7	73,9	2,2

D0	D4	D7	D10	D13-14	Analysis		
					%γδ T cells	%IFN-γ$^+$	%IL-17$^+$
+ IL-7	+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23			0,7	7,9	0,5
			+ IL-7 + IL-1β + IL-23		3,8	4,3	0,5
			+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23	2,9	13,1	0,8
+ IL-7	+ IL-7 + IL-1β + IL-23 + αIFN-γ	+ IL-7 + IL-1β + IL-23 + αIFN-γ			0	0	0
		+ IL-7 + IL-1β + IL-23 + αIFN-γ	+ IL-7 + IL-1β + IL-23 + αIFN-γ		13,4	1,2	0,2
+ IL-7	+ IL-7 + IL-1β + IL-23 + αGL3	+ IL-7 + IL-1β + IL-23 + aGL3			0	0	0
		+ IL-7 + IL-1β + IL-23 + αGL3	+ IL-7 + IL-1β + IL-23 + αGL3		12,3	1,0	0,6
+ IL-15	+ IL-15	+ IL-15	+ IL-15		0	52,8	0
			+ IL-15	+ IL-15	0	0,0	0
+ IL-15	+ IL-15	+ IL-7 + IL-15	+ IL-7 + IL-15		7,1	2,6	9,5
			+ IL-7 + IL-15	+ IL-7 + IL-15	0,0	0,0	0,0
+ IL-7 + IL-15	+ IL-7 + IL-15	+ IL-15			0,2	4,2	1,4
		+ IL-15	+ IL-15		0,3	29,8	1,6
			+ IL-15	+ IL-15	0,0	0,0	0,0
+ IL-7 + IL-15	+ IL-7 + IL-15	+ IL-7 + IL-15			1,4	16,0	0,0
		+ IL-7 + IL-15	+ IL-7 + IL-15		2,4	20,7	0,5
			+ IL-7 + IL-15	+ IL-7 + IL-15	2,0	31,0	0,6
+ IL-7 + IL-1β + IL-23	IL-15	IL-15			1,6	13,6	1,6
		IL-15	IL-15		1,2	45,7	4,3
			IL-15	IL-15	0,2	68,4	10,5
+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-12	+ IL-7 + IL-12			0,8	31,0	0,0
		+ IL-7 + IL-12	+ IL-7 + IL-12		2,2	12,6	3,5
			+ IL-7 + IL-12	+ IL-7 + IL-12	0,8	27,7	0,6
+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23	IL-15	IL-15		2,6	24,7	2,4
			IL-15	IL-15	0,7	37,8	0,4
+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-12	+ IL-7 + IL-12		5,9	9,2	1,8
			+ IL-7 + IL-12	+ IL-7 + IL-12	6,9	13,9	1,0
+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-12 + IL-15	+ IL-7 + IL-12 + IL-15			1,1	22,6	0,5
		+ IL-7 + IL-12 + IL-15	+ IL-7 + IL-12 + IL-15		0,5	59,1	0,8
			+ IL-7 + IL-12 + IL-15	+ IL-7 + IL-12 + IL-15	0,5	73,5	0
+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-12 + IL-15	+ IL-7 + IL-12 + IL-15		6,7	14,0	1,0
			+ IL-7 + IL-12 + IL-15	+ IL-7 + IL-12 + IL-15	4,1	28,2	2,0
+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-15	+ IL-7 + IL-15	7,2	0,2	0,9
+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23			1,6	13,0	0
		+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23		4,6	6,8	2,3
			+ IL-7 + IL-1β + IL-23	+ IL-7 + IL-1β + IL-23	6,4	5,4	2,0

'IL-7*' refers to a cytokine concentration of 10 ng/mL instead of 1 ng/mL.