# A key role for microRNAs in the development and functional differentiation of γδ T cell subsets

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## Introduction

The ability of murine γδ T cells to rapidly produce the pro-inflammatory cytokines interleukin-17 (IL-17) or interferon-γ (IFN-γ) underlies their crucial and non-redundant roles in several (patho)physiological contexts, such as tissue homeostasis, infection, autoimmunity and cancer. This capacity stems from a complex process of 'developmental pre-programming' in the thymus, after which a large fraction of γδ T cells migrate to peripheral sites already committed to producing IL-17 or IFN-γ, unlike their αβ T cell counterparts[1]. So far, several miRNAs have been implied in the control of the differentiation and IFN-γ and IL-17 levels by αβ Th1 and Th17 cells, respectively[2]. However, little is known about the action of these post-transcriptional regulators on γδ T cell differentiation. The finding by Schmolka *et al*. that miR-146a is selectively enriched in IL-17-biased CD27$^-$ γδ T cells and restricts their co-production of IFN-γ by targeting *Nod1* mRNA, therefore regulating γδ T cell plasticity[3], illustrates the need of a more comprehensive study of the miRNA repertoires of γδ T cells and of the regulatory networks they take part in the control of IFN-γ and IL-17 production by these cells.

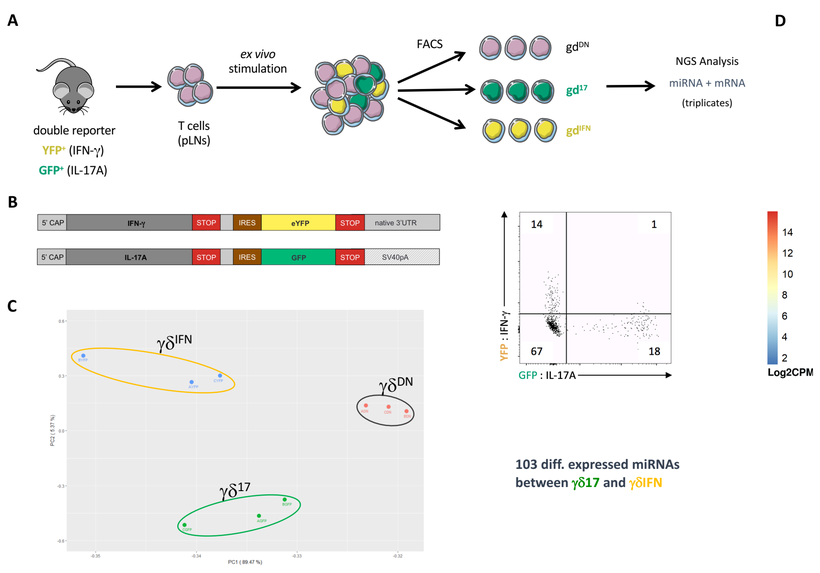
## Aims

To characterize the miRNA:mRNA regulatory networks that regulate IFN-γ and IL-17 expression in γδ T cells subsets *in vivo*:

1. Identify the miRNA and mRNA repertoires of pure IL-17- and IFN-γ-producing γδ T cells;
2. Determine the functional impact of specific miRNAs on γδ T cell differentiation;
3. Analyse the regulation of candidate miRNA expression in IFN-γ$^+$ and IL-17$^+$ γδ T cells;
4. Identify mRNA networks controlled by candidate miRNAs.

## Results

### 1. miRNA and mRNA profiling of IFN-γ$^+$ and IL-17$^+$ γδ T cells



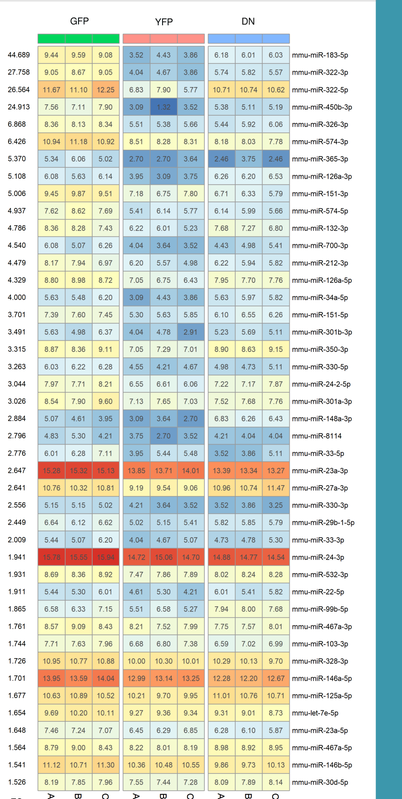




**Figure 1: Differential expression of miRNAs and mRNA between IFN-γ- and IL-17-expressing γδ T cells.** **(A** to **B)** Schematic illustration of the isolation of YFP+-IFN-γ- and GFP$^+$-IL-17-producing γδ T cells and and YFP$^-$GFP$^-$-DN γδ T cells from the peripheral lymph nodes of double reporter IFN-γ.YFP-IL-17A.GFP mice strain in a C57BL/6 background**. (C)** Principal component analysis (PCA) of the miRNA repertoires of YFP$^+$-IFN-γ$^+$, GFP$^+$-IL-17$^+$ and YFP$^-$GFP$^-$-DN γδ T cells. **(D)** Heatmaps depicting differentially expressed miRNAs between IFN-γ$^+$ and IL-17$^+$ γδ T cells. Colors indicate the direction and magnitude of relative expression, with red representing miRNAs an higher expression (Log2CPM). Values inside squares refer to calculated z-scores.

### 2. miRNA:mRNA interaction networks

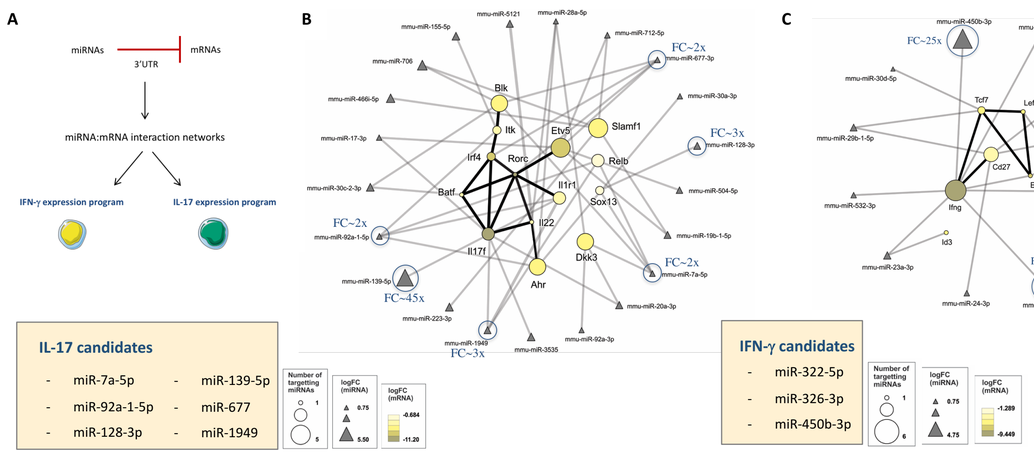



**Figure 2. miRNAs predicted to target key determinant mRNAs of the IL-17 and IFN-γ gene expression networks of γδ T cells.** (**A**) miRNAs differentially expressed in IFN-γ$^+$ or IL-17$^+$ γδ T cells were bioinformatically integrated with mRNAs with a 3' UTR binding site described to be key determinants 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 (**B**) or IFN-γ (**C**) 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. Candidate miRNA overexpression during *in vitro* γδ T cell expansion




**Figure 4. Candidate miRNA overexpression during *in vitro* γδ T cell expansion.** Workflow (**A**) and results (**B** to **C**) of retroviral (RV) overexpression of miR-322, miR-326, miR-450b, miR-7a, miR-92a, miR-128, miR-139 and miR-1949 in peripheral γδ T cells. Flow cytometry analysis of intracellular IL-17 and IFN-γ expression in IL-17$^+$, IFN-γ$^+$ and IL-17$^+$IFN-γ$^+$ γδ T cells and frequency of IL-17$^+$, IFN-γ$^+$ and IL-17$^+$IFN-γ$^+$ in GFP$^+$ retrovirally transduced γδ T cells expressing either a control vector (RV-control) or each candidate miRNA (RV-miR). MFI stands for mean fluorescence intensity. Data are representative of three independent experiments. *$P$ < 0.05 and **$P$ < 0.01.

### 4. miR-128-3p and miR-139-5p restrict IFN-γ production in peripheral γδ T cells

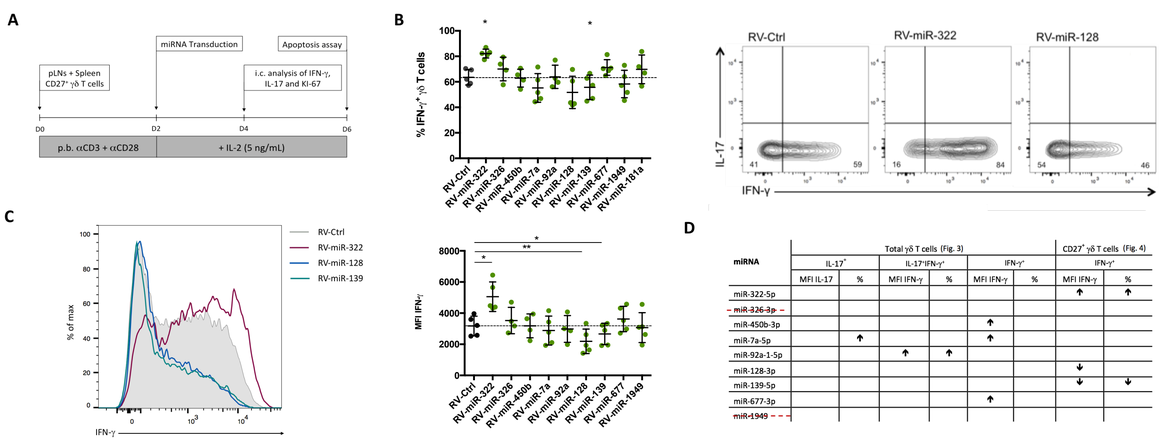



**Figure 4. miR-128 and miR-139 restrict IFN-γ production in peripheral γδ T cells.** Workflow (**A**) and results (**B** to **C**) of retroviral (RV) overexpression of miR-322, miR-326, miR-450b, miR-7a, miR-92a, miR-128, miR-139 and miR-1949 in peripheral CD27$^+$ γδ T cells. Flow cytometry analysis of intracellular IFN-γ expression IFN-γ$^+$ γδ T cells and frequency of IFN-γ$^+$ cells in GFP+ retrovirally transduced γδ T cells expressing either a control vector (RV-control) or each candidate miRNA (RV-miR). (**D**) Effects of candidate miRNA overexpression on cytokine expression of peripheral γδ T cells cultured in vitro; data from Figures 3 and 4. Data are representative of three independent experiments. *P < 0.05 and **P < 0.01.

### 5. miR-128-3p and miR-139-5p have opposite expression patterns along ontogeny

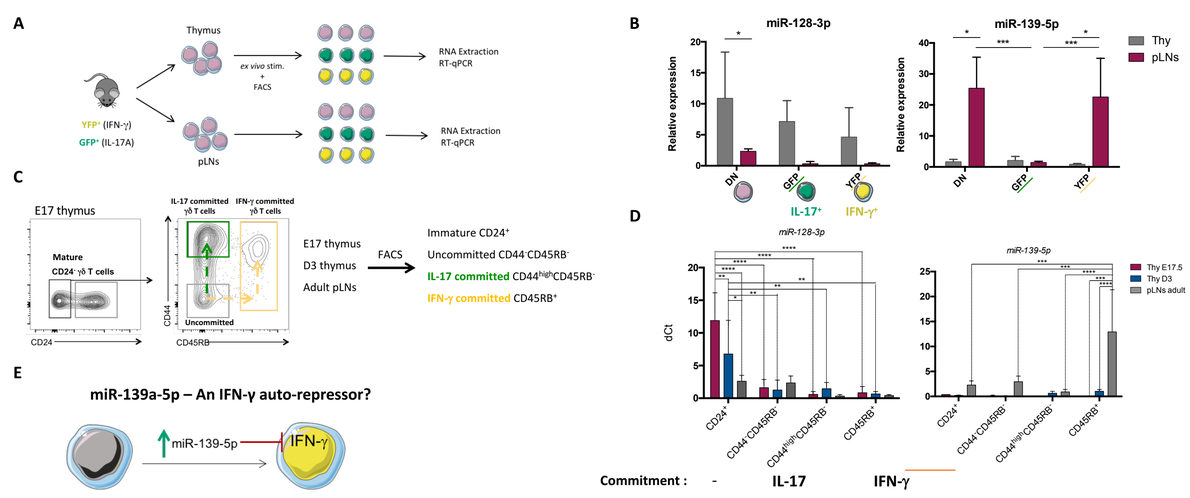



**Figure 5. miR-128-3p and miR-139-5p have opposite expression patterns along ontogeny.** (A) Workflow and (B) results of RT-qPCR analysis of miR-128-3p and miR-139-5p expression in double negative GFP$^-$YFP$^-$, IL-17-producing GFP$^+$ and IFN-γ-producing YFP$^+$ γδ T cell subsets isolated from thymus and peripheral lymph nodes of adult mice. (C) Workflow and (D) results of RT-qPCR analysis of miR-128-3p and miR-139-5p expression in immature CD24$^+$, uncommitted CD44$^-$CD45RB$^-$, IL-17 committed CD44$^{high}$CD45RB$^-$ and IFN-γ-committed CD45RB$^+$ γδ T cell subsets from embryonic E17.5 thymus, neonatal D3 thymus and adult peripheral lymph nodes. (E) miR-139-5p working model based on its detailed pattern of expression throughout γδ T cell ontogeny and in the in vitro gain-of-function studies in γδ T cell cultures. (F) Overall resumed working model for all candidate miRNAs similar to (E). Data are representative of at least three independent experiments. *$P$ < 0.05 and **$P$ < 0.01.

### 6. miR-128-3p controls thymic γδ T cell commitment and differentiation *in vivo*

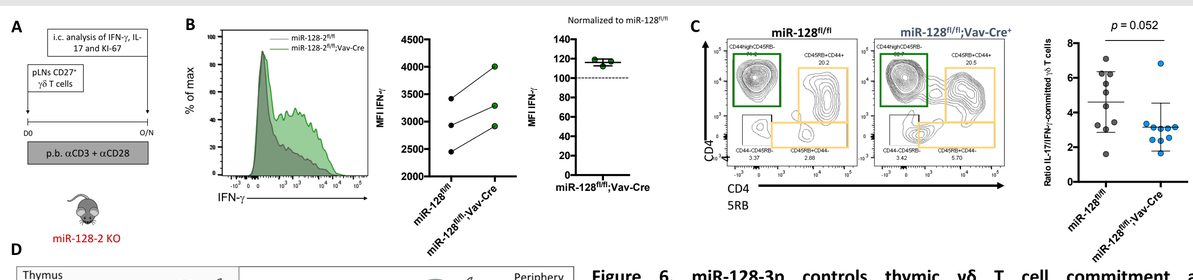



**Figure 6. miR-128-3p controls thymic γδ T cell commitment and differentiation *in vivo*.** Workflow (**A**) and results (**B**) of overnight TCR-stimulation with plate-bound anti-CD3 and anti-CD28 of peripheral CD27$^-$ γδ T cells sorted from the lymph nodes of a hematopoietic-restricted miR-128-2 deficient mouse strain and littermate controls. Flow cytometry analysis of intracellular IFN-γ expression. (**C**) Flow cytometry analysis of mature CD24$^-$ γδ T cell for IL-17 or IFN-γ commitment in the thymus of adult miR-128-2 deficient mice versus littermate controls. (**D**) miR-128-3p working model: in the thymus, where miR-128-3p is highly expressed in γδ T cell precursors, miR-128-3p limits commitment of γδ T cells to the IFN-γ pathway, promoting IL-17 commitment instead. In the periphery, miR-128-3p also inhibits differentiation of γδ T cells into IFN-γ producers in response to TCR stimulation.

### 7. miR-181-5p controls thymic γδ T cell commitment and differentiation *in vivo*

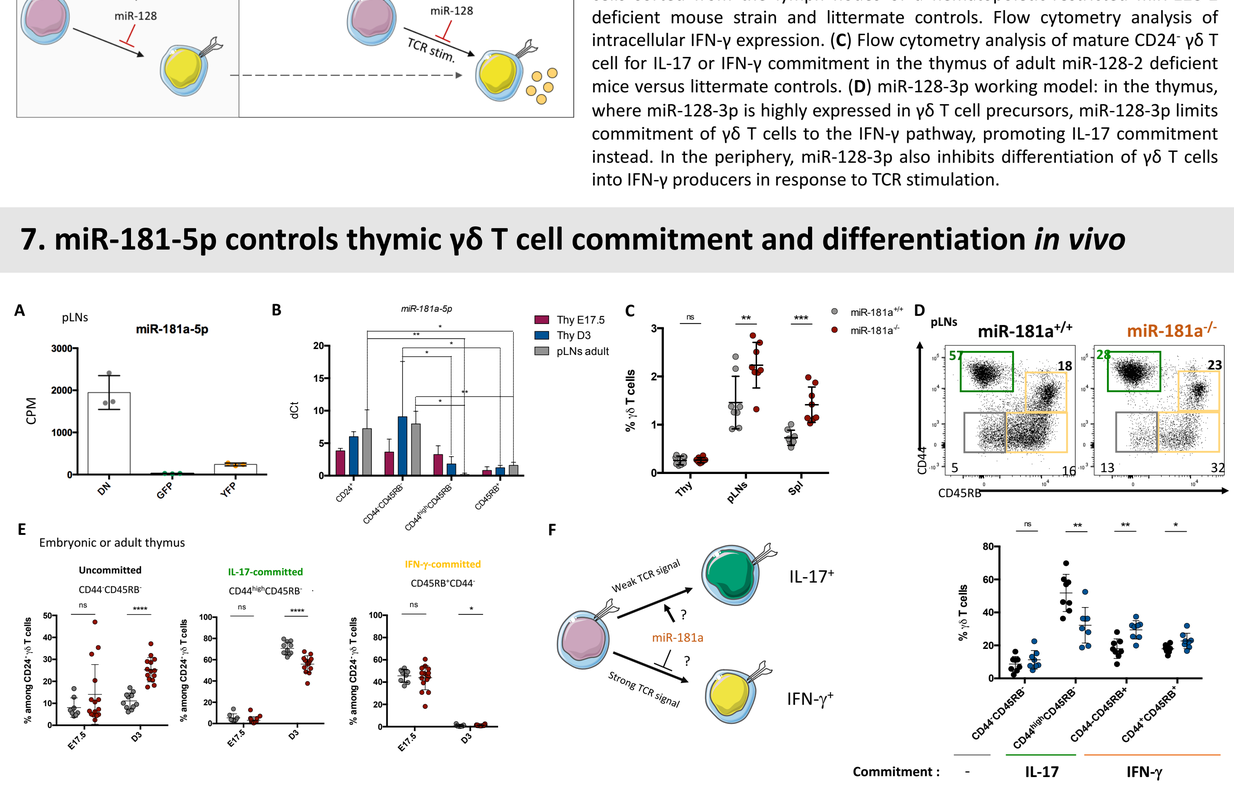



**Figure 7. miR-181-5p controls thymic γδ T cell commitment and differentiation *in vivo*.** (**A**) miR-181a-5p expression in YFP$^-$GFP$^-$DN, GFP$^+$-IL-17$^+$ and YFP$^+$-IFN-γ$^+$ γδ T cells from the RNA-sequencing in Figure 1, in counts per million (CPM). (**B**) RT-qPCR analysis of miR-181-5p expression in immature CD24$^+$, uncommitted CD44$^-$CD45RB$^-$, IL-17 committed CD44$^{high}$CD45RB$^-$ and IFN-γ-committed CD45RB$^+$ γδ T cell subsets from embryonic E17.5 thymus, neonatal D3 thymus and adult peripheral lymph nodes. (**C**) Frequency of γδ T cells in the thymus, peripheral lymph nodes and spleen of adult miR-181a deficient mice and littermate controls identified as CD3ε$^+$TCRδ$^+$ among live cells by flow cytometry. (**D**) Flow cytometry analysis of mature CD24$^-$ γδ T cell for IL-17 or IFN-γ commitment in the peripheral lymph nodes of adult miR-128-2 deficient mice versus littermate controls. (**E**) Flow cytometry analysis of mature CD24$^-$ γδ T cell for IL-17 or IFN-γ commitment in the thymus of embryonic or neonatal miR-128-2 deficient mice versus littermate controls. (**F**) miR-128-3p working model: in the thymus, miR-181-5p limits commitment of γδ T cells to the IFN-γ pathway, promoting IL-17 commitment instead, possibly by inhibiting strong TCR signals that promote IFN-γδ commitment. *$P$ < 0.05, **$P$ < 0.01, *** $P$ < 0.001 and $P$ < 0.0001.

## References

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## Open questions/future work

1. Does **miR-139** function as an IFN-γ auto-repressor *in vivo*?
2. What is the γδ T cell phenotype of embryonic/neonatal **miR-128** KO mice?
3. Which **molecular cues induce** candidate miRNA expression?
4. What are the **relevant mRNA targets** of the candidate miRNA?

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