MicroRNA-Mediated Post-Transcriptional Regulation of Peripheral γδ T Cell Effector Functions

Daniel Inácio[1], Tiago Amado[1], Daniel Sobral[2], Francisco Enguita[3], Carolina Cunha[1], Anita Q. Gomes[1,4*] and Bruno Silva-Santos[1,3*]

[1]Gulbenkian Institute for Molecular Medicine, Lisbon, Portugal; [2]Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisbon, Portugal; [3]Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal; [4]H&TRC Health & Technology Research Center, Escola Superior de Tecnologia da Saúde, Instituto Politécnico de Lisboa, Lisbon, Portugal
*Equal contributions.
daniel.inacio@gimm.pt

Introduction

γδ T cells are unconventional lymphocytes able to rapidly produce large quantities of interleukin-17A (IL-17A) or interferon-γ (IFN-γ), which often have major effects on the pathophysiology of diseases such as cancer or infections. Yet, we do not fully understand how the thymic-derived effector γδ T cell subsets making either IL-17A (γδ^{17} cells) or IFNγ (γδ^{IFN} cells) are activated and function in peripheral tissues. Given the crucial role for microRNAs (miRNAs) in regulating αβ T cell differentiation and effector functions, with several specific miRNAs implied in the control of IFN-γ orIL-17 production by Th1 and Th17 cells, respectively, we set out to dissect the miRNA-mediated post-transcription regulation of peripheral γδ T cell effector functions. To do so, we established an *Il17a-GFP:Ifng-YFP* double-reporter mouse strain to analyse at unprecedented depth the miRNA transcriptomes of pure γδ^{17} versus γδ^{IFN} cell populations from peripheral lymph nodes (pLN), and uncover new mechanisms of miRNA-mediated regulation of effector γδ T cell physiology.

Aims

1. Characterization of subset-specific **IL-17⁺** versus **IFN-γ⁺ γδ T cells microRNA expression profiles;**
2. Uncover **novel microRNA** mediators that control the **selective peripheral activation of IL-17⁺ versus IFN-γ⁺ γδ T cells.**

Results

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

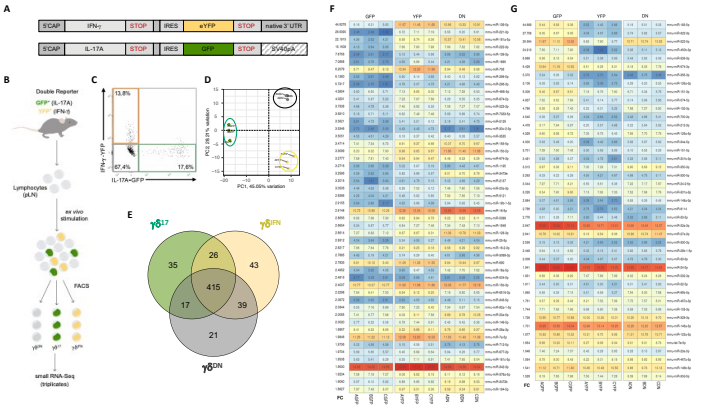

Figure 1. Characterization of the miRNomes of γδ^{17} versus γδ^{IFN} cells. (A-B) Schematic illustration of the isolation of γδ^{DN} (YFP$^-$GFP$^-$), γδ^{17} (GFP$^+$YFP$^-$) and γδ^{IFN} (YFP$^+$GFP$^-$) cell subsets from the pLN of a double reporter IFN-γ.YFP-IL-17A.GFP mouse strain in a C57BL/6 background. **(C)** Representative plot of the proportion of GFP$^+$ and YFP$^+$ γδ T cells obtained by FACS. **(D)** Principal component analysis (PCA) of the miRNA repertoires of γδ^{DN}, γδ^{17} and γδ^{IFN} cell subsets. **(E)** Venn diagram of expressed miRNAs (defined as having a normalized CPM value of >1 in at least two of three samples of the group) in the different subsets. **(F-G)** Heatmaps depicting differentially expressed miRNAs between γδ^{17} and γδ^{IFN} cell subsets, with top overexpressed miRNAs in γδ^{IFN} cells in (F) and top overexpressed miRNAs in γδ^{17} cells in (G). Colors indicate the direction and magnitude of relative expression, with red representing miRNAs an higher expression (Log$_2$CPM). Values inside squares refer to calculated z-scores.

2. miRNA:mRNA interaction networks

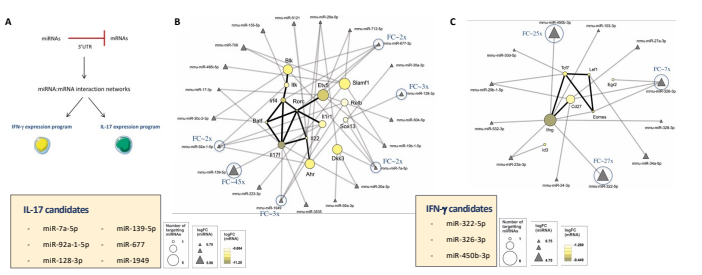

Figure 2. miRNAs predicted to target key determinant mRNAs of the IL-17 and IFN-γ gene expression networks in γδ T cells. (A) miRNAs overexpressed in γδ^{17} or γδ^{IFN} cells were bioinformatically integrated with downregulated mRNAs in the same subset described to be key determinants of the expression of the opposite cytokine on the basis of miRNA-mRNA bioinformatic targeting prediction via 3' UTR. From these we obtained several 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 number and relevance of its predicted target(s) for the respective functional gene program.

3. Effects of candidate miRNA overexpression on IFN-γ production by γδ T cells

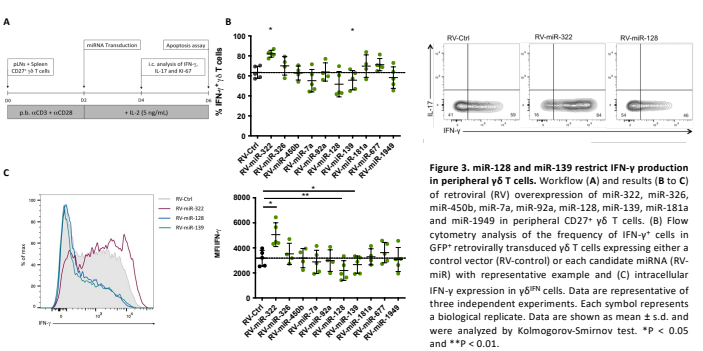

Figure 3. miR-128 and miR-139 restrict IFN-γ production in peripheral γδ T cells. Workflow (**A**) and results (**B** to **C**) of retroviral (RV) overexpression of miR-322, miR-326, miR-450b, miR-7a, miR-92a, miR-128, miR-139, miR-181a and miR-1949 in peripheral CD27$^+$ γδ T cells. (B) Flow cytometry analysis of the 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) with representative example and (C) intracellular IFN-γ expression in γδ^{IFN} cells. Data are representative of three independent experiments. Each symbol represents a biological replicate. Data are shown as mean ± s.d. and were analyzed by Kolmogorov-Smirnov test. *P < 0.05 and **P < 0.01.

4. miR-181a promotes γδ^{IFN} cell effector functions during malaria infection

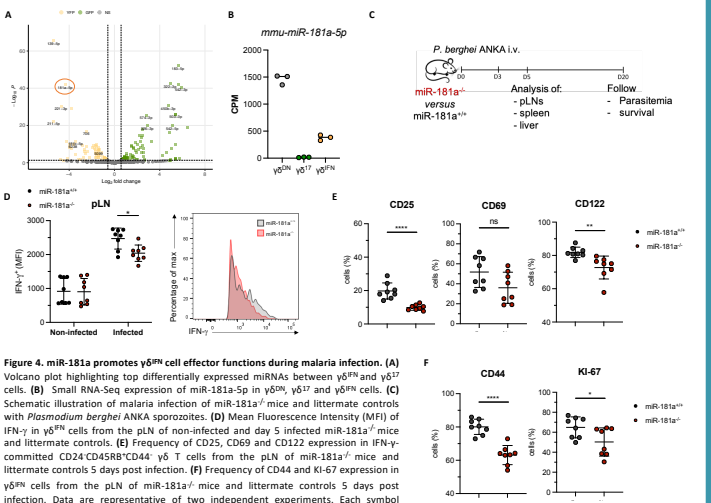

Figure 4. miR-181a promotes γδ^{IFN} cell effector functions during malaria infection. (A) Volcano plot highlighting top differentially expressed miRNAs between γδ^{IFN} and γδ^{17} cells. **(B)** Small RNA-Seq expression of miR-181a-5p in γδ^{DN}, γδ^{17} and γδ^{IFN} cells. **(C)** Schematic illustration of malaria infection of miR-181a$^{-/-}$ mice and littermate controls with *Plasmodium berghei* ANKA sporozoites. **(D)** Mean Fluorescence Intensity (MFI) of IFN-γ in γδ^{IFN} cells from the pLN of non-infected and day 5 infected miR-181a$^{-/-}$ mice and littermate controls. **(E)** Frequency of CD25, CD69 and CD122 expression in IFN-γ-committed CD24$^-$CD45RB^{lo}CD44$^+$ γδ T cells from the pLN of miR-181a$^{-/-}$ mice and littermate controls 5 days post infection. **(F)** Frequency of CD44 and KI-67 expression in γδ^{IFN} cells from the pLN of miR-181a$^{-/-}$ mice and littermate controls 5 days post infection. Data are representative of two independent experiments. Each symbol represents a biological replicate. Data are shown as mean ± s.d. and were analyzed by Mann-Whitney test; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001.

5. miR-181a promotes γδ^{IFN} cell effector functions in response to TCR stimulation

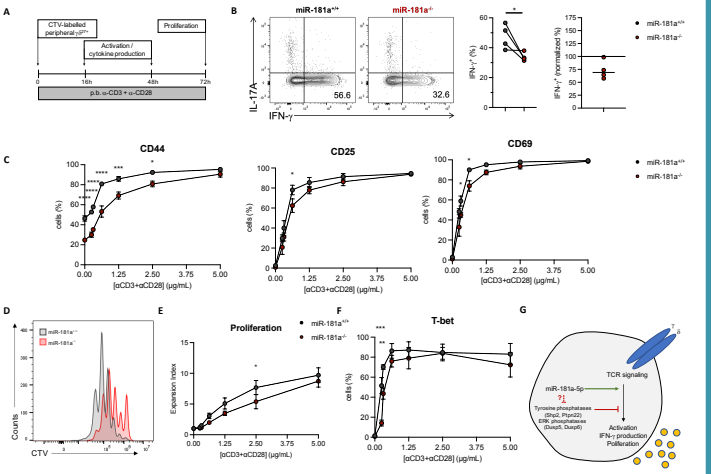

Figure 5. miR-181a promotes γδ^{IFN} cell effector functions in response to *in vitro* TCR stimulation. (A) Schematic illustration of *in vitro* culture system of CTV-labelled CD27$^+$ γδ T cells from pooled pLN and spleen of miR-181a$^{-/-}$ mice and littermate controls with increasing concentrations of plate-bound anti-CD3 plus anti-CD28 for 16h, 48h or 72h. **(B)** Frequency of IFN-γ production by miR-181a-deficient and -sufficient cells cultured for 16h with 2.5 μg/mL of anti-CD3 plus anti-CD28 in the presence of PMA (25 ng/mL), ionomycin (1 μg/mL) and brefeldin A (1 μg/mL) in the last 3h of culture. **(C)** Frequency of CD44, CD25 and CD69 expression in miR-181a-deficient and -sufficient cells cultured for 48h. **(D)** Representative plot of CTV dilution profile of miR-181a-deficient and -sufficient cells cultured for 72h with 2.5 μg/mL of anti-CD3 plus anti-CD28 and **(E)** quantified expansion index. **(F)** Frequency of T-bet expression in intracellularly stained miR-181a-deficient and -sufficient cells cultured for 72h. **(G)** Working model for miR-181a in γδ^{IFN} cells. Data are representative of three to four independent experiments. Each symbol represents a biological replicate (B) or the mean (C,E,F). Data were analyzed by Mann-Whitney (B) two-way ANOVA (C,E,F) and mixed effects model (g and h), under the assumption of data normality, for multiple comparisons. Survival; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001.

Open questions/future work

1. Do **miR-128** and **miR-139** act as **IFN-γ autorepressors** *in vivo*?
2. What are the targets of miR-128, miR-139 and miR-181a in γδ T cells?

References

1. Inácio D., Amado T., Silva-Santos B., Gomes A.Q. (2018) Control of T cell effector functions by miRNAs. *Cancer Lett.*, 427:63-73;
2. Baumjohann D., Ansel K. M. (2013) MicroRNA-mediated regulation of T helper cell differentiation and plasticity. *Nat. Rev. Immunol.*, 13(9): 666–678;
3. Inácio D., Amado T., Pamplona A., (...), Gomes A.Q., Silva-Santos B. (2025) Signature cytokine-associated transcriptome analysis of effector γδ T cells identifies subset-specific regulators of peripheral activation. *Nat. Immunol.*, 26: 497–510.

European Research Council · FCT Fundação para a Ciência e a Tecnologia · República Portuguesa · "la Caixa" Foundation

The project leading to these results has received funding from "la Caixa" Foundation under the project code LCF/PR/HR24/52440006