microRNA-122 and microRNA-1247 regulate the pathogenic phenotype of effector CD4+T cells in (auto)immune responses *in vivo*

Carolina Cunha[1*], Paula Vargas Romero[1], Cataria Pelicano[1], Ana Teresa Pais[1], Daniel Inácio[1], Pedro Papotto[1], Daniel Sobral[2], Tiago Amado[1], Anita Quintal Gomes[1,3*] and Bruno Silva-Santos[1*]

[1]Instituto de Medicina Molecular - João Lobo Antunes (iMM-JLA), Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal
[2]Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal
[3]H&TRC Health & Technology Research Center, ESTeSL - Superior de Tecnologia da Saúde de Lisboa, Instituto Politécnico de Lisboa, Lisboa, Portugal

*anitagomes@medicina.ulisboa.pt

Introduction

MicroRNAs are an abundant class of evolutionarily conserved small non-coding RNA species that control gene expression at the post-transcriptional level and are estimated to regulate most of developmental and physiological processes[1]. They are involved in many aspects of immunity, notably, in the differentiation and function of CD4+ T cell subsets[2]. CD4+ T cells play critical roles in the host defense against pathogens, but they are also responsible for the generation of immune-mediated diseases[3]. This arises from an incorrect balance between the various CD4+ T cell subsets, namely pro-inflammatory effector T cells, including the IFN-γ-producers T helper 1 (Th1) cells and the IL-17-producers Th17 cells, and anti-inflammatory regulatory T cells (FoxP3+ subset)[4]. Although various individual miRNAs have been implicated in CD4+ T cell biology, data is missing on how miRNA regulation networks may control the balance between effector and regulatory T cells in pathophysiological conditions *in vivo*.

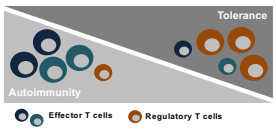

Figure 1: Schematic representation of the immunological balance between effector and regulatory CD4+ T cells. Excessive immune responses and/or deficiency in tolerogenic mechanisms can lead to autoimmune diseases and chronic inflammation. On the other hand, unbalanced induction of regulatory T cells may result in immunodeficiency and persistent infections.

Aim

To dissect the role of miRNAs in the post-transcriptional regulation of effector and regulatory CD4+ T cells differentiation *in vivo*.

Experimental model and methods

1. Establishment of triple *Foxp3-hCD2.Ifng-YFP.Il17a-GFP* reporter mice

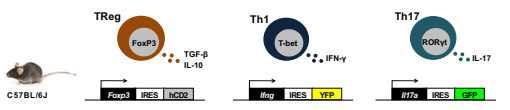

Figure 2: Schematic representation of regulatory (TReg) and effector (Th1 and Th17) populations in triple *Foxp3-hCD2.Ifng-YFP.Il17a-GFP* reporter mice.

2. EAE induction in triple *Foxp3-hCD2.Ifng-YFP.Il17a-GFP* reporter mice

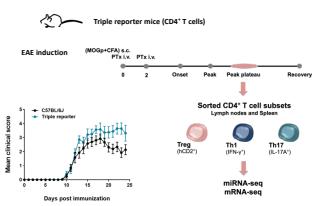

Figure 3: Strategy of EAE induction and comparison of EAE progression between WT and triple *Foxp3-hCD2.Ifng-YFP.Il17a-GFP* reporter mice.
(A) Experimental setup. WT and triple *Foxp3-hCD2.Ifng-YFP.Il17a-GFP* reporter mice were immunized s.c. in both flanks with 100 µg of myelin oligodendrocyte glicoprotein (MOG_{35-55}) peptide emulsified in CFA solution (day 0). On the day of immunization and 2 days later, mice received 200 ng of pertussis toxin i.v. in the eye.CD4+T cells were sorted at the peak plateau phase and RNA extracts of Treg, Th1 and Th17 cells were subject to both mRNA and small-Seq experiments
(B) Mice were weighed daily and scored for EAE clinical signs. Unlike immunized WT mice, triple reporter mice develop a peak plateau stage and rarely recovered.

Results

1. A set of 110 miRNAs are differentially expressed between effector and regulatory T cells *in vivo*

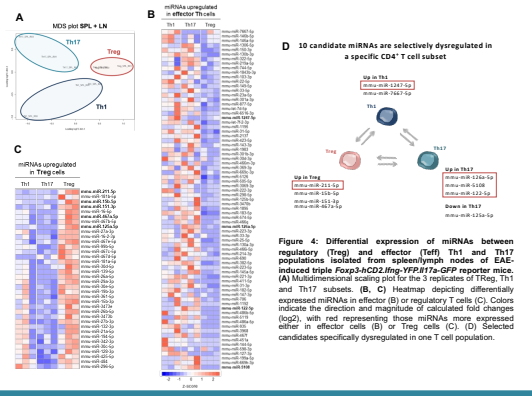

Figure 4: Differential expression of miRNAs between regulatory (Treg) and effector (Teff) Th1 and Th17 populations isolated from spleen/lymph nodes of EAE-induced triple *Foxp3-hCD2.Ifng-YFP.Il17a-GFP* reporter mice.
(A) Multidimensional scaling plot for the 3 replicates of TReg, Th1 and Th17 subsets. **(B, C)** Heatmap depicting differentially expressed miRNAs in effector (B) or regulatory T cells (C). Colors indicate the direction and magnitude of calculated fold changes (log2), with red representing those miRNAs more expressed either in effector cells (B) or Treg cells (C). (D) Selected candidates specifically dysregulated in one T cell population.

2. Treatments with antagomiRs-122 and miR-1247 impact EAE progression

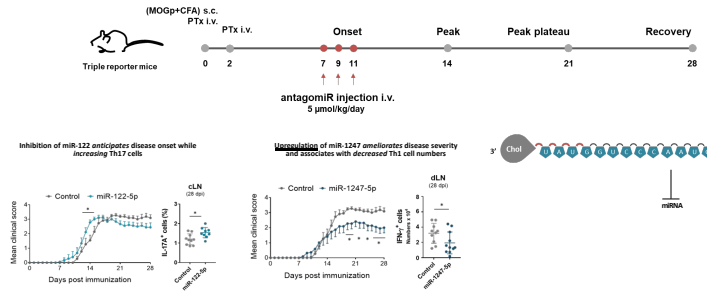

Figure 5 Functional analysis of two candidates using antagomiRs *in vivo* (EAE model). AntagomiRs were designed for miR-122 (up in Th17), miR-1247 (up in Th1) and control antagomir **(A)** Experimental setup. Triple *Foxp3-hCD2.Ifng-YFP.Il17a-GFP* reporter mice were immunized s.c. in both flanks with 100 µg of $(MOG)_{35-55}$ peptide. On the day of immunization and 2 days later, mice received 200 ng of pertussis toxin i.v. in the eye. At days 7, 9 and 11, mice received 5 µmol/kg of specific antagomiRs i.v. in the eye. Cel-miR-67-3p was used as control. **(B)** Mice were scored for EAE clinical signs for 28 days. (C) FACS plots analysis of IFN-γ and IL-17 expression levels in either cervical or draining cLN or dLN of mice subject to miR-1247 and miR-122 antagomiRs.

3. Cytokines modulate miR-122 and miR-1247 expression levels

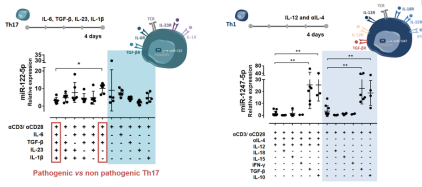

Figure 6. Expression profile of miR-122 and miR-1247 in CD4+ T cells stimulated with different cytokine cocktails. RT-qPCR analysis of miR-122 (A) and miR-1247 (B) expression in CD4+ T cells cultured *in vitro* for 4 days in the presence of indicated cytokines and antibodies.

4. miR-122 and miR-1247 are downregulated in Th17 or Th1 cells, respectively, isolated from the CNS

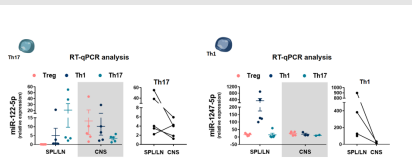

Figure 7. Expression levels of miR-122 and miR-1247 in spleen and CNS. RT-qPCR analysis of miR-122 (A) and (B) miR-1247 expression in CD4+ T cells isolated from the spleen and lymph nodes (SPL/LN) and the central nervous system (CNS) of triple *Foxp3-hCD2.Ifng-YFP.Il17a-GFP* reporter mice challenged with EAE.

Summary

miR-122 and miR-1247 control the pathogenic phenotype of effector Th subsets during CNS autoimmunity

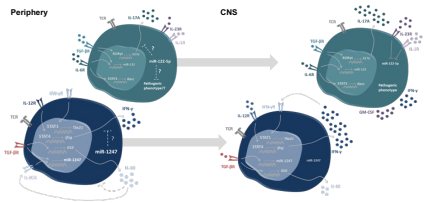

Figure 8. Schematic representation of the working hypothesis.
In the periphery, miR-122 is highly expressed in Th17 cells and negatively regulates IL-17A levels and their pathogenic phenotype. Once Th17 cells invade the CNS and face the inflammatory environment, IL-23 and IL-1β inhibit the expression of miR-122 expression and the negative regulation is subverted allowing Th17 cells to acquire pathogenic properties characterized by the prodution IFN-γ and GM-CSF as observed in our mRNA-seq data (not shown).
Regarding Th1 cells, we hypothesized that miR-1247 is induced by anti-inflammatory cytokines to limit the expression of IFN-γ and prevent excessive inflammation in the periphery. In the CNS, this self-regulation mechanism is no longer in place, miR-1247 levels decrease and Th1 cells produce higher levels of IFN-γ (not shown) contributing to damage the CNS.

Future work

- Identification of miR-122 and miR-1247 target mRNAs via differential Ago2-IP followed by RNA-seq
- Assessment of miR-122 and miR-1247 expression in Multiple Sclerosis' patients samples

References

[1] Mehta, A. and Baltimore, D. (2016). "MicroRNAs as regulatory elements in immune system logic". Nature Reviews Immunology 16: 279-294; [2] Sethi, A.; Kulkarni, N.; Sonar, S. and Lal, G. (2013). "Role of miRNAs in CD4 T cell plasticity during inflammation and tolerance". Frontiers in Genetics 4:8; [3] Jager, A. and Kuchroo, V. (2010). "Effector and regulatory T-cell subsets in autoimmunity and tissue inflammation". Scandinavian Journal of Immunology 72(3):173-84; [4] DuPage, M. and Bluestone, J. (2016). "Harnessing the plasticity of CD4+ T cells to treat immune-mediated disease". Nature Reviews Immunology 16(3):149-63.