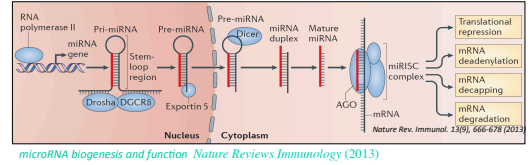


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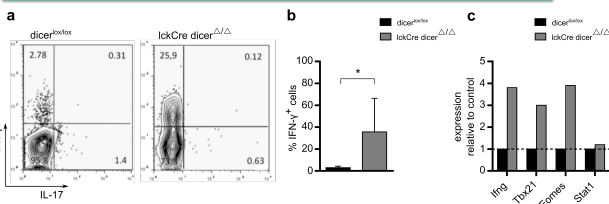
Introduction

MicroRNAs are small non-coding RNA molecules that shape gene expression at the post-transcription level. They have emerged as important players in T cell biology by modulating the development, activation, differentiation and function of these cells. CD8⁺ T cells are key players in immunity against intracellular infections, namely viral infections, and tumors. The main cytokine associated with these protective responses is interferon- γ (IFN- γ), whose production is known to be regulated at the transcriptional level during CD8⁺ T cell differentiation. In this study we screened miRNAs that segregated with IFN- γ expression *ex vivo*, in CD8⁺ T cell subpopulations isolated from an IFN- γ reporter mouse model, and manipulated their activity in parallel *in vitro* and *in vivo* assays. This led us to identify miR-181a as a novel negative regulator of IFN- γ expression in CD8⁺ T cells, whose absence enhanced CD8⁺ T cells ability to respond and control viral infection *in vivo*.



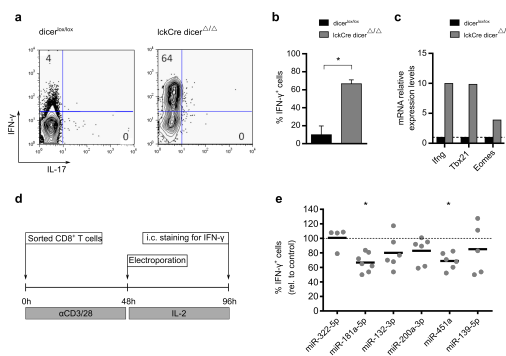
Results

A - Dicer-deficient CD8⁺ thymocytes overexpress IFN- γ



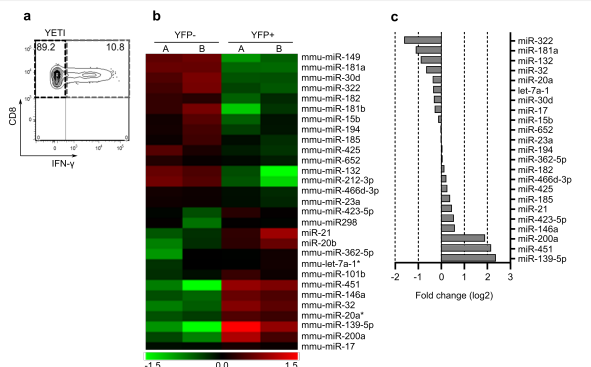
(a) Representative plot (a) and quantification (b) of flow cytometry analysis of IFN- γ and IL-17 protein levels in thymic CD8⁺ T cells of dicerlox/lox and lckCre dicer Δ/Δ mice (n = 4) upon *ex vivo* stimulation with PMA and ionomycin for 4 h at 37 °C with the addition of Brefeldin A. (c) RT-qPCR analysis of Ifng, Tbx21 and Eomes in thymic CD8⁺ T cells of dicerlox/lox relative to lckCre dicer Δ/Δ mice, normalized to housekeeping Actb mRNA. * P \leq 0.05

C - miR-181a-5p and miR-451a limit IFN- γ production by peripheral CD8⁺ T cells



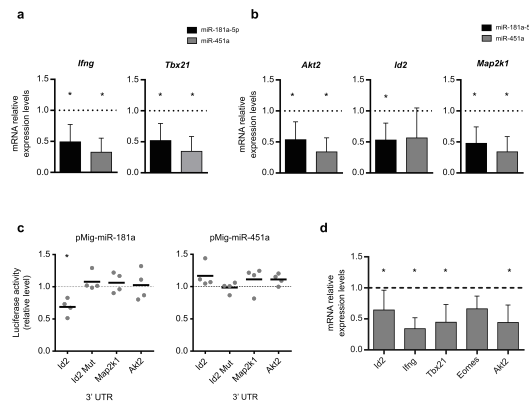
Representative plot (a) and quantification (b) of flow cytometry analysis of IFN- γ and IL-17 protein levels in peripheral CD8⁺ T cells of dicerlox/lox and lckCre dicer Δ/Δ (n = 4). (c) RT-qPCR analysis of Ifng, Tbx21 and Eomes in peripheral CD8⁺ T cells of dicerlox/lox relative to lckCre dicer Δ/Δ mice, normalized to housekeeping Actb mRNA. (d) Representation of the workflow followed for electroporation of miRNA mimics. Quantification (e) of flow cytometry analysis of IFN- γ production by CD8⁺ T cells after electroporation with indicated miRNA mimics (n = 5 to 7). Values in (e) are relative to those of each experiment control condition where a nontargeting negative control miRNA Mimic was transfected. * P \leq 0.05

B - Differential expression analysis of microRNAs segregating with IFN- γ expressing CD8⁺T cell



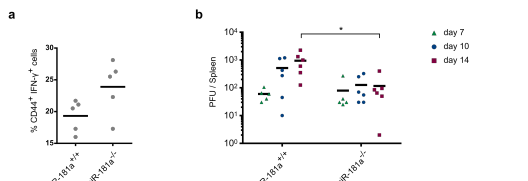
(a) Isolation by FACS of YFP⁻ vs YFP⁺ thymic CD8⁺ T cells from YETI mice for qPCR profiling. (b) Heatmap of differentially expressed genes between YFP⁻ and YFP⁺ CD8⁺ thymic T cells. The color scale shown at the bottom illustrates the relative expression level of a miRNA across all samples; red color represents an expression level above mean, and green color represents expression lower than the mean. (c) qPCR validation of selected miRNA genes' differential expression between IFN- γ -producing vs nonproducing thymic CD8⁺ T cells. miRNAs enriched in YFP⁻ have negative log₂ fold change values while miRNAs enriched in YFP⁺ have log₂ fold change positive values

D - miR-181a-5p and its mRNA target conversely regulate Ifng expression in CD8⁺ T cells



RT-qPCR analysis of (a) signature genes Ifng and Tbx21 and (b) miR-181a-5p target genes Akt2, Id2, and Map2k1 upon transfection of cells with either miR-181a-5p or miR-451a mimics (n = 4). Values are relative to those of each experiment control condition where a nontargeting negative control miRNA Mimic was transfected. (c) Luciferase reporter assay to verify interaction between overexpressed microRNAs, miR-181a or miR-451a, and 3' UTRs of selected targets: Id2, Map2k1 or Akt2. Mutation of miR-181a-5p binding sites in Id2 3' UTR followed by luciferase assay was performed to prove miR-181a binding specificity. Renilla/luciferase ratios were normalized to those obtained for empty pMig construct (n = 4). (d) RT-qPCR analysis of Id2, Ifng, Tbx21, Eomes, and Akt2 genes after siRNA-mediated Id2 knockdown (n = 4). Values are relative to those of each experiment control condition where a nontargeting negative control siRNA was transfected. * P \leq 0.05

E - Lack of miR-181-5p enhances antiviral CD8⁺T cell responses *in vivo*



miR-181a^{-/-} and miR-181a^{+/+} littermates were infected intranasally with 10⁴ PFU of murid herpesvirus 4 (MuHV-4). (a) Percentages of activated (CD44⁺) CD8⁺ T cells expressing IFN- γ as assessed by intracellular staining. (b) Viral loads (PFU) quantified by *ex vivo* reactivation assays in which latently infected splenocytes (harvested at days 7, 10 and 14 postinfection) were co-cultured with permissive BHK-21 cells. Each dot represents one mouse. Horizontal lines indicate the mean. * P \leq 0.05

Summary

- Dicer-deficient thymic and peripheral CD8⁺ T cells overexpress IFN-g
- CD8⁺ T cell electroporation with miR-181a or miR-451a mimics reduces IFN-g production *in vitro*
- miR-181a inhibits IFN-g production by targeting Id2
- Id2 knock-down phenocopies miR-181a overexpression in CD8⁺ T cells
- miR-181a limits anti-viral IFN-g producing CD8⁺ T cell responses *in vivo*