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Research paper

miR-21 is a negative modulator of T-cell activation

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ABSTRACT

microRNAs (miRNAs) are a class of small non-coding RNAs acting as post-transcriptional regulators of gene expression and play fundamental roles in regulating immune response and autoimmunity.

We show that memory T-lymphocytes express higher levels of miR-21 compared to naïve T-lymphocytes and that miR-21 expression is induced upon TCR engagement of naïve T-cells. We identify *bona fide* miR-21 targets by direct immuno-purification and profiling of AGO2-associated mRNAs in Jurkat cells over-expressing miR-21. Our analysis shows that, in T-lymphocytes, miR-21 targets genes are involved in signal transduction. Coherently, TCR signalling is dampened upon miR-21 over-expression in Jurkat cells, resulting in lower ERK phosphorylation, AP-1 activation and CD69 expression. Primary human lymphocytes in which we impaired miR-21 activity, display IFN-γ production enhancement and stronger activation in response to TCR engagement as assessed by CD69, OX40, CD25 and CD127 analysis. By intracellular staining of the endogenous protein in primary T-lymphocytes we validate three key regulators of lymphocyte activation as novel miR-21 targets.

Our results highlight an unexpected function of miR-21 as a negative modulator of signal transduction downstream of TCR in T-lymphocytes.

1. Introduction

Recent studies pinpoint miRNAs as major players in the molecular circuitries that control development and differentiation of haematopoietic lineages. miRNAs are a class of small non-coding RNAs which modulate gene expression at post-transcriptional level [1]. These tiny RNA molecules are incorporated into the RISC (RNA Induced Silencing Complex) by tight association with Argonaute (AGO1-4) proteins. RISC exerts its regulatory function through impairment of target mRNA translation and/or mRNA destabilization [1]. Computational predictions suggest that each single miRNA could affect the expression of hundreds of proteins [2]. Several reports highlighted key roles for miRNAs in the development and function of lymphocytes [3].

TCR stimulation triggers proliferation and activation of naïve T-lymphocytes by a complex signal transduction cascade, finally resulting in activation of NFAT, AP-1, NFkB and other transcription factors. The subsequent wave of mRNA transcription and protein translation leads to the differentiation of resting naïve T-cells into effector and memory T-lymphocytes. Sub-threshold signals may result in anergy, apoptosis or low expansion of memory cells which are unable to mount a proper immune response.

We show that miR-21 is highly expressed in human central memory T-lymphocytes compared to naïve T-cells. This is consistent with previous reports describing miR-21 expression in memory $CD8^+$ cells in mice [4], in lymphocytes of patients affected by Systemic Lupus Erythematosus [5] as well as in lymphocytes of PD-1 knock-out mice [6]. In mice miR-21 is a limiting factor of immune response [7]. A tumorigenic role has also been highlighted for this miRNA [8]. Nevertheless, an extensive characterization of miR-21 targets is lacking and mechanistic details of its mode of action in T-lymphocytes are still unknown. Identification of miRNA targets is mandatory for molecular characterization of their function. Canonical targets are recognized by pairing between their 3′ UTR and

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a short region of 6–8 nucleotides at the 5′ end of the miRNA, termed *seed* [9]. Several bioinformatic approaches have been developed to predict miRNA targets [10]. Recently, biochemical purification of miRNA targets via RISC/miRNA pull down has been exploited [11,12]. This approach unveils that a relevant fraction of miRNA targets lack canonical seed complementarity and are missed by most prediction algorithms.

We demonstrated that miR-21 expression in primary human T-lymphocytes affects TCR signalling. By an unbiased high-throughput biochemical approach, we identified specific interactions between miR-21 and a large group of mRNAs encoding for key players of TCR signal transduction and lymphocyte activation. We validate three of the identified targets by intracellular staining of endogenous protein in primary T-cells. Our results show that miR-21 expression affects ERK phosphorylation, AP-1 activity and T-cell activation and interleukins (IFN-γ) production upon TCR engagement, highlighting a role of miR-21 as a negative regulator of T-lymphocyte activation.

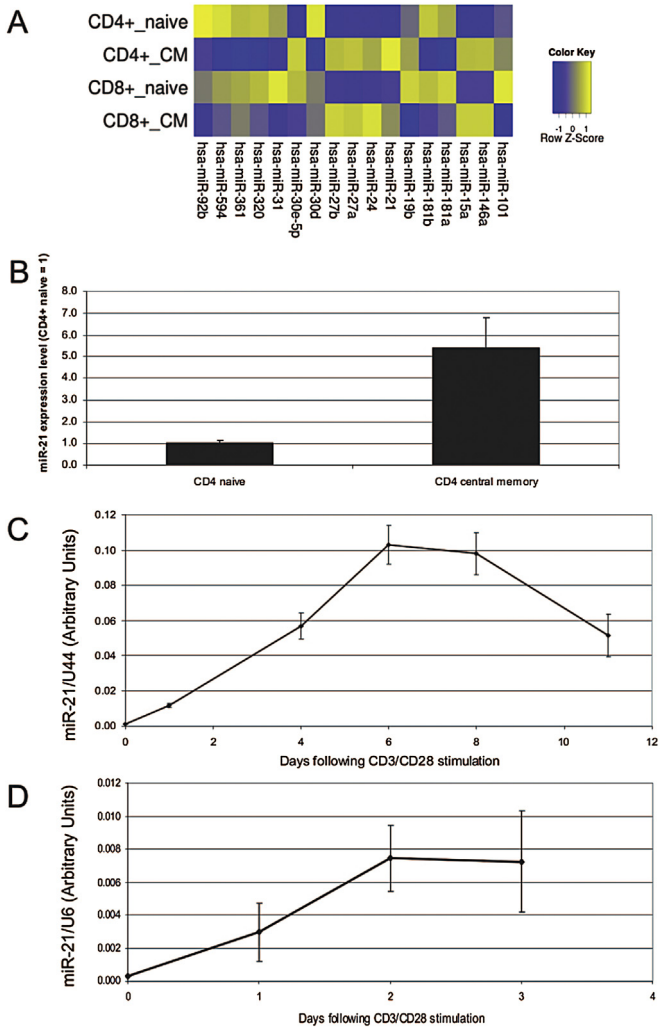

Fig. 1. miR-21 is expressed at higher levels in central memory T-lymphocytes and is induced upon TCR engagement of T-lymphocytes in vitro. A) Heatmap of miRNA expression profile of T-lymphocytes ($CD4^+$ naïve, $CD4^+$ memory, $CD8^+$ naïve and $CD8^+$ central memory cells) from peripheral blood of healthy donors. B) miR-21 expression was quantified by qRT-PCR in $CD4^+$ naïve and central memory cells of healthy donors. C) miR-21 expression in T-lymphocytes following stimulation by anti CD3/CD28 antibodies. D) miR-21 expression is specifically induced in the T-ALL cell line Jurkat upon treatment with anti CD3/CD28 antibodies.

2. Materials and methods

2.1. Microarray data

microRNA microarray assay was performed using a service provider (LC Sciences). GEO repository accession: GSE16453.

Primary $CD4^+$ lymphocytes were assayed for gene expression by using the Affymetrix Human Gene 1.0 ST array (3 replicates). GEO repository accession: GSE37213.

Details are provided in supplementary experimental procedures.

2.2. qRT-PCR of mature miRNAs

qRT-PCR of mature miR-21 was performed as described previously [13].

2.3. Lentiviral transduction

Viral particles were obtained by co-transfection of 293T-cells with lentiviral plasmid and the PLP-1, PLP-2 and PLP-VSVG plasmids (Invitrogen) and concentrated by ultra-centrifugation. Jurkat cells were transduced at an MOI of 15 and selected with puromycin.

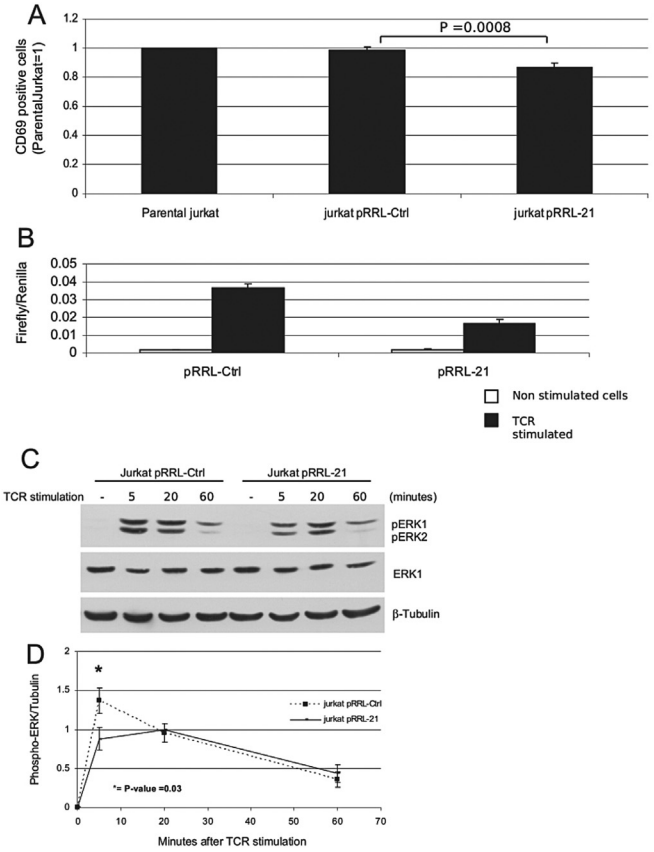

Fig. 2. Jurkat cells overexpressing miR-21 are less responsive to TCR stimulation. A) Assessment of CD69 positive cells 24 h after CD3/CD28 stimulation; Jurkat_pRRL-Ctrl and Jurkat_pRRL-21 cells are compared to parental Jurkat cells. Average of three independent experiments is reported. Error bars represent SEM. B) AP-1 reporter activity is depicted for Jurkat_pRRL-Ctrl and Jurkat_pRRL-21, non stimulated and CD3/CD28 stimulated cells were analysed. Average of three independent replicas is reported. Error bars represent SEM. C) Western blot analysis of ERK phosphorylation. Cells were stimulated and harvested at the indicated time-points. One representative experiment of four independent replicas is shown. D) Densitometric analysis of four independent replicas of phosphoERK western blot.

Primary CD4$^+$ cells were activated using Invitrogen anti CD3/CD28 Dynabeads and transduced at an MOI of 15 with polybrene.

2.4. Jurkat cell activation

Culture plates were incubated O/N at 4 °C with a polyclonal rabbit anti-mouse immunoglobulins (Dako). 150 μl of αCD3 (okt-3) and αCD28 were added. The plates were incubated for 4 h at 37 °C. Cells were plated on the Ab-coated wells and incubated for the indicated times.

2.5. AP-1 reporter assay

Cells were cotransfected with 3 μg of AP-1 reporter construct and 1 μg of the pRLTK plasmid using Lipofectamine 2000 (Invitrogen), according to manufacturer instructions. Following transfection cells were split, in one well the cells were activated with CD3/CD28 antibodies, while the other well was used as a non-activated control. Luciferase activity was assessed using Dual Luciferase assay (Promega).

2.6. RISC immunopurification

Jurkat cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.5; 150 mM KCl; 0.5% Nonidet P-40; 2 mM EDTA; 0.5 mM DTT; 1 mM NaF; 40 u/ml RNasin). Lysates were clarified and pre-cleared by protein-G sepharose beads. An aliquot of total extract was taken out (Input). Monoclonal anti-AGO2 (11A9, Ascenion) and an equal amount of purified rat IgG (SIGMA) were incubated with the pre-cleared lysate. Samples were washed with lysis buffer and wash buffer (50 mM Tris–HCl, pH 7.5; 300 mM NaCl; 5 mM Mg2Cl; and 0.05% Nonidet P-40), treated with DNaseI-RNase-free (Promega) and subject to proteinase K digestion. After final wash an aliquot was taken out for western.

3. Results

3.1. Differential miRNA expression profile between naïve and memory T-lymphocytes

We looked at the miRNA expression profile of naïve and central memory T-lymphocytes in the CD4$^+$ and CD8$^+$ lineages, sorted

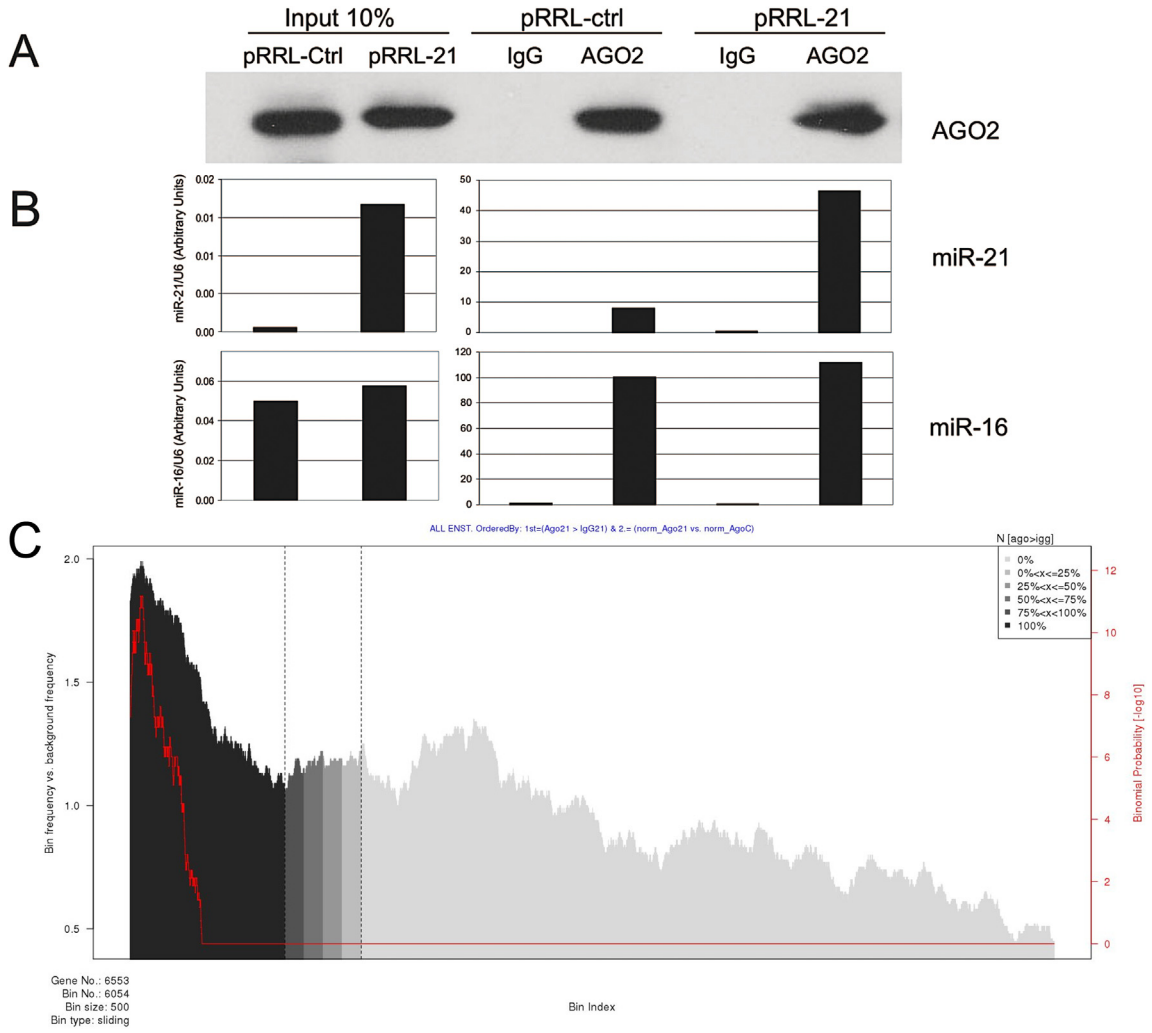

Fig. 3. RISC-IP to identify miR-21 targets in Jurkat_pRRL-Ctrl and Jurkat pRRL-miR-21. A) Western blot of the immunoprecipitated protein, AGO2 and IgG samples. B) miRNA-21 and miRNA-16 abundance was measured by qRT-PCR immunoprecipitates in total lysate, AGO2 and IgG samples. C) Black area: frequency of miR-21 seed within 3′ UTRs of genes specifically bound to RISC (AGO2 signal > IgG signal) in pRRL-21 cells compared to the frequency of miR-21 seed in the expressed mRNAs; light grey area: frequency of miR-21 seed within 3′ UTRs of genes not specifically bound to RISC (AGO2 signal < IgG signal) in pRRL-21 cells compared to the frequency of miR-21 seed in the expressed mRNAs; red line: *P*-value (binomial probability).

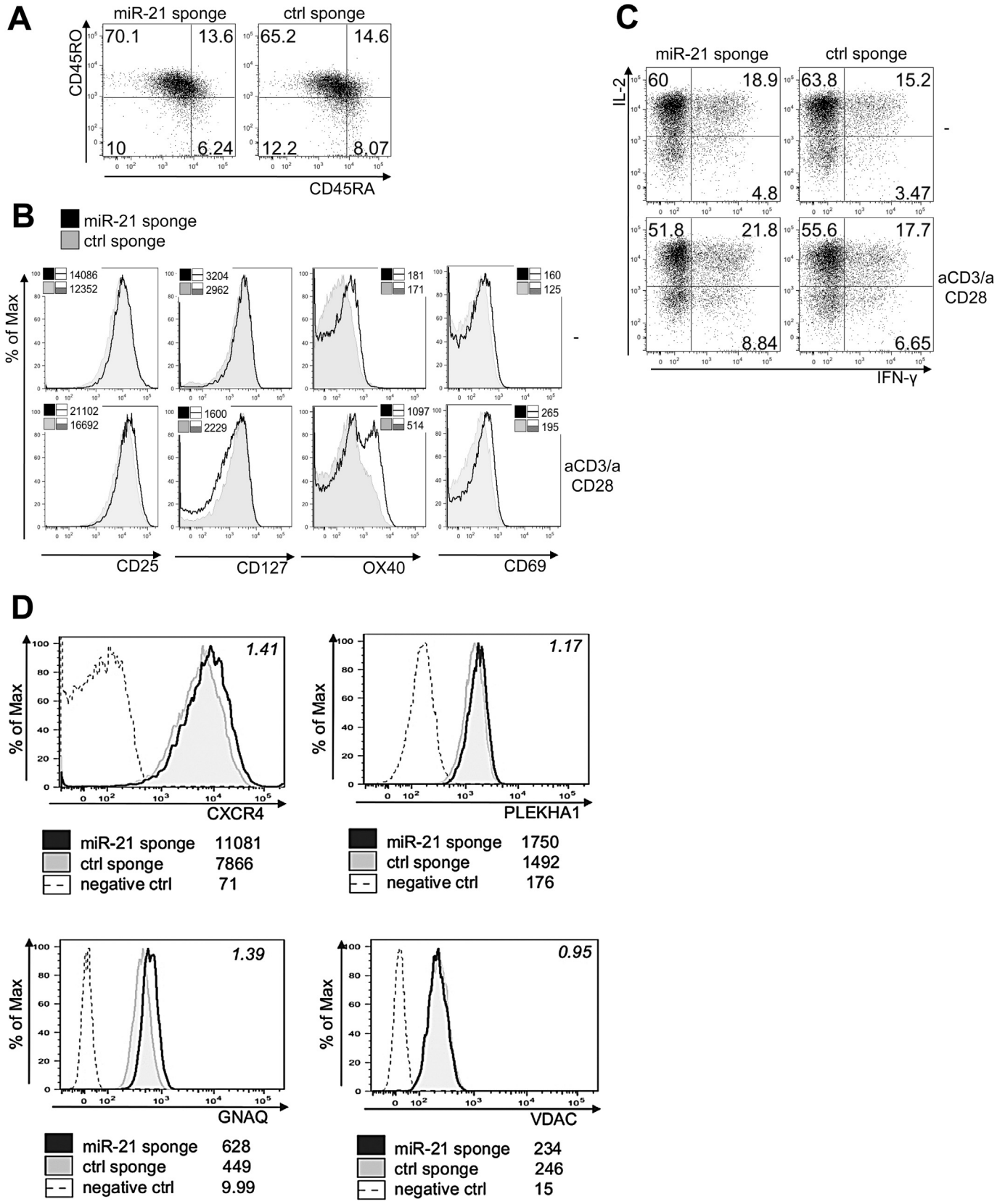

Fig. 4. miR-21 inhibition results in stronger activation of T-lymphocytes. Primary $CD4^+$ lymphocytes were transduced with miR-21_sponge or CTRL_sponge constructs (carrying GFP as reporter), expanded for 6 days with CD3/CD28 Dynabeads and restimulated (or not) overnight with 5 μg/ml of soluble anti-CD3/CD28 antibodies. A) Representative dot plot showing CD45RA versus CD45RO expression. Data show not restimulated, gated GFP^+ T-cells, from one out of 4 independent experiments. B) Histogram overlay of CD25, CD127 and, OX40 and CD69 expression in gated GFP^+ cells. Numbers in each plot represent the mean fluorescence intensity (MFI) of markers. Data are representatives of one of 3 independent experiments. C) Intracellular staining of IL-2 and IFN-γ in gated GFP^+ primary $CD4^+$ lymphocytes transduced with miR-21_sponge or CTRL_sponge constructs, restimulated (or not)

from healthy human individuals as $CD45RA^{+}CD45RO^{-}$ or $CD45RA^{-}CD45RO^{+}CCR7^{+}$ cells, respectively. Overall, $CD4^{+}$ and $CD8^{+}$ naïve cells showed similar profile, as well as $CD4^{+}$ and $CD8^{+}$ central memory cells. A few miRNAs (miR-15a, miR-21, miR-24, miR-27a, miR-27b, miR-146a and miR-101) were differentially expressed in central memory compared to naïve cells in both $CD4^{+}$ and $CD8^{+}$ lineages (Fig.1A).

We focus on miR-21, which was previously shown to act as an anti-apoptotic factor and to be over-expressed in several tumours [8]. qRT-PCR analysis confirmed that central memory $CD4^{+}$ T-lymphocytes express miR-21 at a five-fold higher level compared to naïve cells (Fig.1B).

3.2. miR-21 expression is induced via TCR stimulation and is related to a memory phenotype

To verify whether miR-21 expression is a consequence of TCR engagement, naive $CD4^{+}$ T-lymphocytes from healthy donors were treated with anti-CD3/CD28 agonistic antibodies [14]. We observed an up-regulation of miR-21 (Fig.1C) consistent with what observed during naïve-to-memory transition in $CD4^{+}$ T-lymphocytes. miR-21 expression is detectable within 24 h of stimulation reaching a plateau after 3 days and remaining high afterwards for at least 11 days. Similar results were obtained using PMA/ionomycin, which activate signal transduction downstream of TCR (Supplementary Fig. 1). This process was recapitulated also in Jurkat cells (Fig.1D), a model for the study of TCR signal transduction in a cell line. These data demonstrate that miR-21 expression in T-lymphocytes is triggered by TCR stimulation and costimulation.

3.3. Expression of miR-21 prior to TCR stimulation results in dampened stimulation

We over-expressed miR-21 in Jurkat cells by transduction with a lentiviral transgenic construct encoding for miR-21 under a PGK promoter (pRRL-21) [15]. A cognate vector encoding for a control hairpin was used to generate control cell line (pRRL-Ctrl). Jurkat_pRRL-21 cell line over-expressed miR-21 roughly 20-fold higher compared to Jurkat_pRRL-Ctrl (Supplementary Fig. 2A), this is comparable to the expression attained following TCR/CD28 stimulation in the parental cell line. Jurkat_pRRL-Ctrl express miR-21 at levels comparable to those observed in parental cell line prior to stimulation (Supplementary Fig. 2B).

Since it has been reported that transfection of miRNAs may displace endogenous miRNAs from RISC [13], we checked by deep sequencing the profile of miRNAs loaded on AGO2 (Supplementary Table 2). In Jurkat pRRL-21, AGO2-bound miR-21 was up-regulated without significantly affecting association of other miRNAs with AGO2.

We found that Jurkat_pRRL-21 cells were less responsive than Jurkat_pRRL-Ctrl cells to anti-CD3/CD28 stimulation, we observed a highly reproducible ($P = 0.0008$) decrease in expression of the early activation marker CD69 in pRRL-21 compared to pRRL-Ctrl cells (Fig. 2A).

CD69 expression is driven by several transcription factors downstream of TCR engagement, such as AP-1 and NF-kB [16]. We assessed activity of these transcription factors by using luciferase reporter constructs. No significant change in NF-kB activity was detect (data not shown). On the contrary, Jurkat_pRRL-21 cells, compared to Jurkat_pRRL-Ctrl cells, displayed significantly lower induction of AP-1 reporter activity after TCR stimulation ($P = 0.026$, Fig 2B). AP-1 is a transcription factor downstream of the MAP kinase cascade activated by RAS [17]. We therefore measured ERK phosphorylation, which is also triggered by RAS activation and is an established marker of RAS activity in response to TCR engagement. Fig. 2C–D shows a significant decrease of the ERK phosphorylated forms in pRRL-21 cells compared to pRRL-Ctrl at 5 min after stimulation. These data confirm that miR-21 expression in T-lymphocytes affects signal transduction downstream of TCR.

3.4. High-throughput biochemical identification of miR-21 targets

We set out to identify miR-21 targets using a high-throughput biochemical approach. Using a monoclonal antibody against AGO2, RNA associated to RISC complexes were immunopurified from pRRL-21 and pRRL-Ctrl Jurkat cells. As a control, we performed a parallel immunoprecipitation using isotype-matched rat IgG (Fig 3A). miRNA abundance was measured by qRT-PCR. High levels of miR-16 (Fig. 3B), let-7f, miR-142-5p (data not shown) were found in both AGO2 immunoprecipitates but not in IgG controls. In agreement with miR-21 up-regulation, high levels of miR-21 were specifically found in AGO2 immunoprecipitates from pRRL-21 cell lysates, and not pRRL-Ctrl AGO2 and IgG controls (Fig 3B). To identify mRNAs specifically recruited onto RISC upon miR-21 over-expression, we profiled AGO2-bound mRNAs from pRRL-21 and pRRL-Ctrl cell lysates (herein referred to as 21_AGO2 and Ctrl_AGO2 samples) and control IgG captured mRNAs (herein referred to as 21_IgG and Ctrl_IgG samples) by microarray analysis.

We compared AGO2-bound mRNAs in pRRL-21 and pRRL-Ctrl cell lines. We used 21_IgG and Ctrl_IgG to discriminate mRNAs aspecifically bound to AGO2 antibody and/or sephadex protein-G beads. For each mRNA the ratio between (21_AGO2/21_IgG)/(Ctrl_AGO2/Ctrl_IgG) was used to rank candidate miR-21 targets (Supplementary Fig. 3 and Supplementary experimental procedures). We computed frequencies of different miRNA *seeds* in the 3′ UTRs of those mRNAs specifically associated to RISC in pRRL21 cells. We found a significantly higher frequency of miR-21 *seed* matches within 3′ UTRs of mRNAs bound to RISC only in pRRL-21 cells when compared to the general frequency among the expressed mRNAs (*P*-value $< 1\ E{-}10$, Fig 3C). No significant increase in *seed* frequency was observed in the same 3′ UTRs for miRNAs which are highly expressed in both pRRL-21 and pRRL-Ctrl cells or miRNAs which are not expressed in these cell lines. These observations confirm that our approach effectively identifies *bona fide* miR-21 targets.

3.5. Functional annotation enrichment analysis (GO terms) highlights a role of miR-21 targets in key process for lymphocyte activation and differentiation

We performed a GO term analysis [18] to identify the pathways in which mRNAs bound by miR-21 are involved. We ranked mRNAs according to their binding to RISC specifically in pRRL-21 cells and submitted the list to a GO term enrichment analysis tool [19]. The enriched Biological Process GO term categories (Supplementary Fig. 4) were related to GTP metabolism, regulation of lymphocyte development and cell differentiation. Strikingly, Molecular Function Annotation (Supplementary Fig. 4A) yielded only categories

overnight with anti-CD3/CD28 and activated 4 h with Cell Stimulation Cocktail plus protein transport inhibitors. Data are representative of 4 independent experiments. D) FACS validation of *bona fide* miR-21 targets. miR-21_sponge and CTRL_sponge cells were surface stained with anti-CXCR4 Ab, or intracellularly stained with anti-PLEKHA1, anti-GNAQ or anti-VDAC primary Abs. As negative control, unstained cells (for CXCR4) or cells stained with secondary Ab alone are shown. Numbers in each legend show MFI, while numbers in each plot represent the ratio between MFI in miR_21 sponge and MFI in CTRL_sponge. Data are representative of more than three independent replicates.

related to GTPase enzymatic activity, while analysis of Cellular Component (Supplementary Fig. 4B) pointed to cell membrane and vesicles. These data indicate a role of miR-21 in the inhibition of small GTPases-mediated and membrane-associated signal transduction, a key process for lymphocyte activation and differentiation.

3.6. Analysis of miR-21 role in primary T-lymphocyte activation

We exploited a more physiological system, namely primary naïve $CD4^+$ T-lymphocytes sorted from peripheral blood of healthy donors and activated in vitro. We took advantage of lentiviral transduction. Efficient lentiviral transduction of primary T-lymphocytes requires T-cell activation that is followed by strong miR-21 induction (Fig. 1). We set out to inhibit miR-21 function by using a sponge construct [20], rather than to further increase its expression in activated T-lymphocytes. Eight sequences complementary to miR-21 except for a bulge (nt 12–13), were cloned in a lentiviral vector downstream of a GFP reporter, thus obtaining the miR-21_sponge construct [15]. A cognate vector containing a control sequence was used as a control (CTRL_sponge). Efficient titration of miR-21 by miR-21_sponge construct was assayed in Jurkat cells (Supplementary Fig. 5).

Primary naïve $CD4^+$ $CD45RA^+$ T-cells were activated by CD3/CD28 beads and transduced with the above described lentivirus, five days later cells were re-activated with a milder stimulus (soluble anti-CD3/CD28 antibodies at low concentrations). The extent of cell activation was monitored by flow cytometry. miR-21_sponge expressing cells displayed higher levels of CD45RO acquisition and lower levels of CD45RA downregulation, suggesting that miR-21 inhibition results in a faster differentiation toward a memory phenotype (Fig 4A). We also highlighted higher levels of CD69, CD25, and OX40 expression and lower levels of CD127 expression in miR-21_sponge cells compared to CTRL_sponge cells. In particular, CD25 and OX40 levels were higher both before and after re-stimulation, even though OX40 was induced upon re-stimulation at much higher levels in miR-21_sponge cells (Fig. 4B). On the other hand, CD69 was specifically increased and CD127 specifically decreased in miR-21_sponge cells only upon re-stimulation. Overall the expression pattern of these markers indicates that miR-21 inhibition favours T-cell activation and re-activation.

We also tested cytokine production in miR-21_sponge cells. While IL-2 secretion seemed to be unaffected, we could detect a significant increase in IFN-γ production in miR-21_sponge cells (Fig. 4C).

The above-described alterations were not a mere consequence of increased proliferation/survival. Indeed, the analysis of proliferation rate by E-fluor 670 labelling did not highlight any significant alteration. Despite the reported role of miR-21 as an anti-apoptotic factor, no significant differences were observed when measuring by TUNEL assay apoptosis induction in response to CD3 stimulus in miR-21_sponge and CTRL_sponge cells (data not shown).

3.7. Microarray analysis of miR-21_sponge and CTRL_sponge transduced primary T-lymphocytes

We performed mRNA expression profile of miR-21_sponge and CTRL_sponge transduced cells. *Seed* analysis does not reveal a significant enrichment for miR-21 seed in the mRNAs upregulated in miR-21_sponge samples (Supplementary Fig. 6), suggesting that those are secondary targets of miR-21. Coherently, mRNA expression profiles of Jurkat_pRRL-21 and Jurkat_pRRL-Ctrl did not highlight any bias toward primary miR-21 targets downregulation. However, GO term annotation is consistent with the observed phenotype (Supplementary Fig. 7), highlighting that mRNAs whose expression is affected by miR-21 sponge are involved in immune response.

3.8. Validation of biochemically identified miR-21 targets

We confirm our biochemical target identification approach by intracellular flow cytometric analysis of endogenous protein in miR-21_sponge compared to CTRL_sponge primary $CD4^+$ T-cells. We chose a set of *bona fide* targets for validation which have previously been involved in regulation of T-lymphocyte activation (PLEKHA1 [21], GNAQ [22], RAC1 [23], OGT [24] and CXCR4 [25]) and another set of targets not implicated in T-lymphocyte activation (RAB14 and VDAC). We were able to specifically stain PLEKHA1, GNAQ, CXCR4 and VDAC, while we could not obtain sufficient specificity (compared to negative controls) for RAC1, OGT and RAB14. This analysis shows that three of four genes selected were up-regulated in T-lymphocytes upon miR-21 inhibition (Fig. 4D).

4. Discussion

miR-21 expression in T-lymphocytes was reported in association with autoimmune diseases such as SLE [5] and psoriasis [26], as well as in Sézary syndrome [27]. Interestingly, silencing of miR-21 ameliorates splenomegaly in SLE [28]. More recently, the role of miR-21 has been explored in primary human T-lymphocytes showing that miR-21 plays opposing roles in naïve and memory T-cells [29]. Furthermore, transfection of primary murine lymphocytes with miR-21 suggested that this miRNA might boost T-cell activation [30]. However, a loss of function approach in mice has shown that miR-21 limits immune response-mediated activation of the IL-12/IFN-gamma pathway and Th1 polarization [7]. The use of different model systems (ex vivo cultured human and murine primary T-lymphocytes, lymphoblastic cell lines) and, more importantly, different methods (in vivo approaches, transfection, lentiviral transduction) does not allow a direct comparison of these partially conflicting results.

Although a few putative miR-21 targets in T-lymphocytes have been tested, an exhaustive characterization of miR-21 function at molecular level in human T-lymphocytes is still lacking.

We demonstrate that repression of signal transduction by miR-21 occurs upstream of CD69 expression, by showing that both AP-1 activity and ERK phosphorylation are affected by miR-21. Multiple markers of T-lymphocyte activation (CD69, CD25, OX40) are up-regulated in primary T-lymphocytes by specific inhibition of miR-21 activity. We also report higher IFN-γ production by miR-21-depleted primary T-lymphocytes. This phenotype is corroborated by mRNA profile in primary lymphocytes, highlighting deregulation of mRNAs involved in immune response upon miR-21 depletion.

To characterize the role of miR-21 in human T-lymphocytes, we exploited a high-throughput biochemical approach, that allows direct identification of miRNA targets in the cell type of interest. Via lentiviral transduction, we obtained a miR-21 upregulation closely resembling the physiological upregulation observed upon T-cell activation and identified hundreds of *bona fide* miR-21 target mRNAs specifically recruited onto AGO2. mRNA-targets display a significant enrichment for 7-mers sequences complementary to miR-21 *seed* in their 3′ UTRs and a strong enrichment for GO categories associated with signal transduction. miR-21 inhibition in primary T-lymphocytes results in an increased expression of 3 out of 4 measured *bona fide* miR-21 targets (PLEKHA1, CXCR4, GNAQ, VDAC), yielding a validation rate of our *bona fide* miR-21 targets of 75%.

These novel targets point to miR-21 as a negative regulator of signal transduction in T-lymphocytes. We show that miR-21 co-

ordinately affects expression of a large number of targets, while having a mild effect on target mRNA stability. Nevertheless, fine-tuning of a large number of targets converging on few pathways results in a robust and reproducible phenotype.

Our results are in line with the observations by Lu and colleagues [7]. On the contrary, Wang and co-workers [30] obtained different results by miR-21 transfection into murine T-lymphoctes and Jurkat cells. However, it should be noted that in our experimental setting a 20-fold upregulation of miR-21 was obtained via lentiviral transduction in human primary T-lymphocytes, resembling the physiological upregulation of this miRNA that we observe upon T-cell activation. This approach might reflect more closely the mode of action of miRNAs in physiological conditions.

Despite the established role of miR-21 as a negative modulator of apoptosis [31] we did not identify any effect on apoptosis of miR-21 in T-lymphocytes.

miR-21 has been shown to promote AP-1 function in thyroid cells through PDCD4 down-modulation [32]. However, we did not observe any modulation of PDCD4, an explanation might be that T-lymphocytes express a different isoform of PDCD4 which is not targeted by miR-21. In line with the hypothesis that miRNA function is determined by the specific cellular context, we highlight a novel role of miR-21, which rather than enhancing MAP kinase signalling and ERK phosphorylation in lymphocytes is a repressor of signalling through this pathway. Our findings are consistent with the observation that miR-21 limits adaptive immune response and affects IFN-γ production in murine lymphocytes [7].

Based on the observed phenotypes, we suggest that miR-21 expression could play a role in modulating TCR sensitivity of memory T-cells, perhaps to avoid hyper-activation in response to sub-optimal stimulation. Indeed, due to their extremely rapid and strong response to TCR stimulation, sensitivity of TCR signalling should be tightly controlled in memory T-cells, miR-21 may be a candidate for this role. Further investigations will be required to validate this hypothesis.

Conflict of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.09.021.

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