MicroRNA-181a restricts human γδ T cell differentiation by targeting Map3k2 and Notch2

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Abstract

γδ T cells are a conserved population of lymphocytes that contributes to anti-tumor responses through its overt type 1 inflammatory and cytotoxic properties. We have previously shown that human γδ T cells acquire this profile upon stimulation with IL-2 or IL-15, in a differentiation process dependent on MAPK/ERK signaling. Here, we identify microRNA-181a as a key modulator of human γδ T cell differentiation. We observe that miR-181a is highly expressed in patients with prostate cancer and that this pattern associates with lower expression of NKG2D, a critical mediator of cancer surveillance. Interestingly, miR-181a expression negatively correlates with an activated type 1 effector profile obtained from in vitro differentiated γδ T cells and miR-181a over-expression restricts their levels of NKG2D and TNF-α. Upon in silico analysis, we identify two miR-181a candidate targets, Map3k2 and Notch2, which we validate via overexpression coupled with luciferase assays. These results reveal a novel role for miR-181a as a critical regulator of human γδ T cell differentiation and highlight its potential for manipulation of γδ T cells in next-generation immunotherapies.

Keywords cancer; effector T lymphocytes; microRNAs; miR-181a; γδ T cells
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Introduction

Despite the recent advances in therapeutic strategies against cancer, early tumor recurrence and novel metastasis formation indicate resistance to current treatments. This urges the development of alternative treatments for advanced stages of the disease. γδ T cells possess multiple anti-tumor characteristics, making them promising candidates to be used in cellular and combination therapies (Gentiles et al., 2015; Silva-Santos et al., 2015, 2019). They provide IFN-γ-associated type 1 responses against cancer and express critical determinants of tumor cell recognition, including their signature γδ TCR but also a variety of NK cell receptors (NKRs), among which the natural killer group 2 member D (NKG2D) is of utmost importance (Lança et al., 2010; Correia et al., 2013; Silva-Santos et al., 2015; Wu et al., 2019). This notwithstanding, the γδ T cell-based clinical trials completed to date have shown objective responses of only 10–33% (Gomes et al., 2010; Lo Presti et al., 2017). This modest outcome could be explained by their deficient expansion and/or dysregulated effector functions in vivo (Argentati et al., 2003; Bryant et al., 2009; Gaal et al., 2009; Kuroda et al., 2012).

Parallel investigations in both mice and humans have suggested that the tumor microenvironment can subvert the anti-tumor type 1 effector γδ T cell phenotype, either inactivating it (Marten et al., 2006; Gonnermann et al., 2015) or diverting into immunosuppressive phenotypes (Peng et al., 2007; Hao et al., 2011; Ma et al., 2012; Ye et al., 2013a, 2013b) or promoting the expansion of distinct protumor type 17 effector γδ T cells (Rei et al., 2014; Wu et al., 2014; Silva-Santos et al., 2019). Collectively, these limitations stress the need to restore or enhance γδ T cell type 1 inflammatory and cytotoxic properties in future immunotherapeutic approaches.

Based on this background, we have previously demonstrated that the anti-tumor type 1 effector properties of γδ T cells are selectively acquired upon stimulation with IL-2 or IL-15, but not IL-4 or IL-7 (Ribot et al., 2014). The effects of IL-2/IL-15 depended on MAPK/ERK signaling and induced de novo expression of the type 1 transcription factors T-bet and eomesodermin. We followed those studies by exploring an additional layer of regulation of γδ T cell differentiation, namely, post-transcriptional mechanisms mediated by microRNAs (miRNAs or miRs), which are still poorly characterized in γδ T cells (Fiala et al., 2020), especially in the human setting (Zhu et al., 2017).

MiRNAs are naturally occurring and evolutionarily conserved endogenous small non-coding RNAs (18–25 nucleotides) that typically downregulate post-transcriptional gene expression by binding to the 3’ untranslated region (UTR) of their target mRNAs and promoting their degradation or inhibiting their translation (Ambros, 2004). Each precursor miRNA consists of two mature RNA sequences—the 5p and 3p strands—whose designation is attributed according to the directionality of the miRNA strand (Kozomara &
Griffiths-Jones, 2014). Although it has long been proposed that the 5p strand is the one being loaded into the RNA-induced silencing complex (RISC), recent evidence has disproven the idea that the 3p strand is mainly degraded during miR biogenesis (Kozomara et al., 2019). In fact, both the 5p and 3p strands can be loaded onto the Argonaute (AGO) family of proteins in an ATP-dependent manner (Yoda et al., 2010) and can show differential expression levels according to the pathophysiological context under study, namely, in cancer (Mitra et al., 2015, 2020). Importantly, miRNAs exert their regulatory functions in a highly combinatorial way: One miRNA can regulate several mRNAs in parallel, and different miRNAs can target one mRNA simultaneously, thus repressing its expression more efficiently (Pons-Espinal et al., 2017). Since this post-transcriptional process controls the expression of most mammalian genes, it is particularly relevant to analyze its role in human γδ T cell differentiation. To date, only one study has highlighted a role for miR-125B-5p and miR-99a-5p in human γδ T cell activation and cytotoxicity (Zhu et al., 2017), while the involvement of other miRs and their potential impact on γδ T cell responses to cancer remains unclear.

Here, we identify miR-181a as a novel molecular regulator of human γδ T cell functional differentiation. We demonstrate that both its -5p and -2-3p strands control γδ T cell type 1 effector differentiation and responsiveness by targeting Map3k2 and Notch2 mRNAs, and suggest a potential implication of this process in metastatic cancer patients.

**Results and Discussion**

**miR-181a is upregulated in peripheral γδ T cells from metastatic cancer patients**

We investigated potential associations between miRNA expression patterns and γδ T cell dysfunction in metastatic cancer patients compared to healthy controls. Reduced number and impaired pro-inflammatory cytokine production have been previously reported in circulating γδ T cells from patients with melanoma (Argentati et al., 2003), glioblastoma (Bryant et al., 2009), breast (Gaafar et al., 2009), and gastric (Kuroda et al., 2011) carcinomas. Here, we analyzed patients of the cancer types with highest incidence in women (breast) and men (prostate), both in the metastatic (stage IV) setting. We found lower numbers of γδ T cells in the peripheral blood of both cohorts, and a decrease in their expression of NKG2D (significant in the prostate cohort), when compared to the respective (female or male) healthy controls (Fig 1A and B). We also observed a tendency for reduced IFN-γ production in the peripheral γδ T cells from the breast cancer patient cohorts, but this did not reach statistical significance (Fig 1C). Whereas the expression of miR-125B-5p and miR-99a-5p, which have previously been reported to modulate γδ T cell activation and cytotoxicity (Zhu et al., 2017), was very low and comparable to healthy controls (Fig 1D), we found—among other candidates under study—miR-181a-5p and miR-181a-2-3p to be upregulated in metastatic cancer patients, especially in the prostate cancer cohort (Fig 1E), thus providing an interesting association with γδ T cell dysfunction (Fig 1A and B). Of note, the effects observed were independent of patient age and treatment history.

miR-181a is known for its pleiotropic functions on γδ T cell differentiation, including controlling the Th1 response of human CD4+ T cells (Sang et al., 2015); however, the role of miR-181a has never been addressed in human γδ T cells.

**miR-181a is downregulated upon type 1 effector γδ T cell differentiation**

Given each individual’s history of infections, circulating γδ T cells in healthy donors are mostly functionally mature cells (Gibbons et al., 2009; deBarros et al., 2011). By contrast, we have previously shown that human γδ thymocytes isolated from pediatric biopsies of thymus are functionally immature and can be induced to acquire a type 1/cytotoxic profile upon stimulation with IL-2 or IL-15, but not IL-4 or IL-7 (Ribot et al., 2014). Based on our data described above, we hypothesized that miR-181a might be downregulated during type 1 effector differentiation of human γδ thymocytes. Consistent with our hypothesis, we observed that the expression of both miR-181a strands was significantly lower in in vitro differentiated (IL-2 cultured) γδ thymocytes compared to immature (IL-7 cultured) controls (Fig 2A). In fact, all miR-181a species, including pre-miRNAs and mature 5p and 3p strands, were downregulated upon IL-2 treatment (Fig EV1A and B). Moreover, this downregulation of miR-181a (-5p and -2-3p) expression was also found when comparing freshly isolated (immature) γδ thymocytes versus (mature) peripheral γδ T cells ex vivo (Fig 2B).

Given that human γδ T cells comprise two major subsets, Vδ1+ cells, that are more abundant in the thymus and non-lymphoid tissues, and Vδ2+ cells, which represent 60–95% of γδ T cells in the peripheral blood and in lymph nodes (Fichtner et al., 2020), we assessed whether the composition of these populations could influence the levels of miR-181a expression in total γδ T cell samples. Although we found a higher level of miR-181a expression in the Vδ1+ γδ T cell subset (Fig EV2A), the Vδ1/Vδ2 ratio did not correlate with miR-181a expression levels (Fig EV2B). We next anticipated that, besides IL-2, other cytokines might regulate miR-181a expression and thus cultured γδ thymocytes with a set of pro- and anti-inflammatory mediators. IL-7 was used as control condition, allowing cell survival without promoting their functional differentiation (Ribot et al., 2014). Like IL-2, IL-15, which is also known to promote γδ T cell functional differentiation (Ribot et al., 2014), substantially downregulated the expression of both miR-181a strands (Fig 2C). By contrast, TGF-β upregulated miR-181a expression, while other cytokines such as IL-4, IL-12, IFN-γ, and TNF-α showed no impact (Fig 2C). Collectively, these data revealed that miR-181a expression is downregulated by drivers of type 1 effector γδ T cell differentiation, while being induced by immunosuppressive cytokines, namely, TGF-β, which is typically enriched in the cancer setting (Batlle & Massagué, 2019). This may have pathophysiological relevance, as TGF-β significantly enhanced miR-181a expression in γδ T cells isolated from the peripheral blood of healthy donors (Fig 2D), thus mimicking the increased miR-181a levels displayed by γδ T cells from patients with prostate cancer (Fig 1E).

To further document the negative association of miR-181a with type 1 effector γδ T cells, we next analyzed the expression of molecular hallmarks in various γδ T cell samples at different stages of differentiation. Namely, we used the same samples (i.e., freshly isolated versus in vitro IL-7- or IL-2-cultured γδ thymocytes) to measure the expression of miR-181a-5p and -2-3p, as well as the surface protein levels of the type 1 cytotoxic mediator NKG2D, and the
intracellular expression of the type 1 cytokines, IFN-γ and TNF-α. We observed a striking inverse correlation between the expression of both miR-181a strands and the percentages of γδ thymocytes positive for IFN-γ, TNF-α, and NKG2D (Fig 2E), fully consistent with our hypothesis that miR-181a negatively regulates type 1 effector γδ T cell differentiation. Interestingly, this inverse correlation was not sustained in ex vivo peripheral blood γδ T cells (Fig 2F), suggesting a role for miR-181a during the early differentiation process, after which cytokine production becomes constitutive in mature cells. On the other hand, NKG2D showed a more dynamic profile in

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**Figure 1.** Peripheral γδ T cells from metastatic cancer patients display impaired effector functions and increased expression of miR-181a.

A. γδ T cell concentration in the peripheral blood from healthy donors and indicated metastatic cancer patients.
B. RT–PCR analysis of the expression of NKG2D in γδ T cells isolated from indicated samples.
C. RT–PCR analysis of the expression of IFN-γ and TNF-α in γδ T cells isolated from the peripheral blood of healthy controls and metastatic cancer patients.
D. RT–PCR analysis of the expression of miR-125b-5p and miR-99a-5p in γδ T cells isolated from indicated samples.
E. RT–PCR analysis of the expression of miR-181a(-5p and -2-3p) in γδ T cells isolated from indicated samples.

Data information: (A–E) Error bars represent the mean ± SEM. CTRL F = Control Females (n = 25–27 independent biological samples); BC = Breast Cancer (n = 79–83 independent biological samples); CTRL M = Control Males (n = 19–20 independent biological samples); PC = Prostate Cancer (n = 29–36 independent biological samples). Statistical analysis was performed using the unpaired Student's t-test with Welch's correction. ns, not significant. *P < 0.05, **P < 0.01, and ****P < 0.0001. All experiments were performed with two technical replicates.

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Peripheral γδ T cells, which maintained the inverse correlation between NKG2D and miR-181a levels (Fig 2F) as observed in γδ thymocytes (Fig 2E). Consistently, the upregulation of miR-181a expression observed in TGF-β cultured peripheral γδ T cells (Fig 2D) associated with a lower percentage of NKG2D⁺ cells (Fig EV3). While this may suggest a segregation of the molecular mechanisms...
that regulate NKG2D and the pro-inflammatory cytokines in fully mature peripheral γδ T cells, our results collectively pointed toward a role for miR-181a during effector γδ T cell differentiation, which we set out to test using a gain-of-function approach.

**miR-181a overexpression impairs γδ T cell differentiation**

To formally test whether miR-181a is able to regulate effector γδ T cell differentiation, we used retroviral transduction to overexpress miR-181a. We designed a construct in order to overexpress the native stem-loop of miR-181a (containing both -5p and -2-3p strands) in γδ thymocyte cultures. Cells were freshly isolated, activated, transduced, and maintained in the presence of IL-7 and IL-2 to promote type 1 cytotoxic differentiation (Fig 3A). The expression of the GFP reporter allowed us to identify the subpopulation that integrated the retroviral construct (Fig 3B). As a technical control of the GFP reporter allowed us to identify the subpopulation that integrated the retroviral construct (Fig 3B). As a technical control of the GFP reporter allowed us to identify the subpopulation that integrated the retroviral construct (Fig 3B). As a technical control of the GFP reporter allowed us to identify the subpopulation that integrated the retroviral construct (Fig 3B). As a technical control of the GFP reporter allowed us to identify the subpopulation that integrated the retroviral construct (Fig 3B). As a technical control of the GFP reporter allowed us to identify the subpopulation that integrated the retroviral construct (Fig 3B).

On the other hand, as an internal control, we consistently analyzed the untransduced GFP+ population, where miR-181a expression remained at baseline (Fig 3C), and thus, no changes in any of the below-mentioned readouts were expected.

miR-181a overexpression seemingly impaired γδ T cell survival, given the increase in Annexin V+ cells (Fig 3D) and the reduction in expression of the anti-apoptotic genes Bcl2 and Bcl-xl (Fig 3E) among the transduced (but not the untransduced) γδ T cells. On the other hand, miR-181a overexpression increased the percentage of cells with a central memory profile (CD27+CD45RA−), while reducing the percentage of cells displaying a typical naive (CD27−CD45RA+) and effector phenotype (CD27−CD45RA−; Fig 3F). Since previous studies reported that memory T cells arise from the presence of high concentration levels of IL-2 (Berard & Tough, 2002; Yurova et al, 2017), our results support a functional crosstalk between miR-181a and IL-2-dependent signaling.

From a functional standpoint, we demonstrated that miR-181a overexpression led to significant reductions in the percentages of TNF-α and NKG2D+ γδ T cells (Fig 3F). Interestingly, while the percentage of IFN-γ+ γδ T cells was not affected, we observed a decrease in the percentage of TNF-α IFN-γ+ subset, suggesting that, in the type 1 effector differentiation pathway, miR-181a could also regulate a step where IFN-γ production would be induced from the TNF-α population, as previously proposed for Vδ2+ γδ T cells and NK cells (Skeen & Ziegler, 1995; Li et al, 2008). Of note, we observed that miR-181a overexpression equally affected the percentage of TNF-α+ cells within Vδ1+ and Vδ2+ population (Fig EV4).

We further overexpressed miR-181a in mature γδ T cells isolated from healthy donor peripheral blood (Fig EV5A and B) and found a significant albeit minor decrease in NKG2D levels, while type 1 differentiation program remained intact (Fig EV5C). This is consistent with our previous data pointing at an inverse correlation in peripheral γδ T cells between the expression of miR-181a and NKG2D, but not type 1 cytokine expression (Fig 2F). Of note, by comparing GFPlow and GPFhigh subsets, either in γδ thymocyte (Fig EV5D) or PBL (Fig EV5E) cultures, we did not observe any dose effect of the levels of miR-181a-bearing vector transduction. Overall, the differences between PBL and thymocyte cultures suggest that the maturation status of γδ T cells conditions their sensitivity to miR-181a action, which is maximized at early stages of functional γδ T cell differentiation.

**miR-181a targets Map3k2 and Notch2 mRNAs**

To dissect the molecular mechanisms underlying miR-181a function, we went on to identify putative mRNA targets. For this, a bioinformatical analysis using the mirDIP v4.1 database, coupled to a Gene Set Enrichment Analysis (GSEA) of candidates involved in Th1 differentiation and dysregulated in cancer, allowed us to predict a list of candidates based on the miR-181a-5’ and -2-3’ mature sequences, common to both strands (Dataset EV1). Interestingly, we could highlight the Notch and Mapk signaling pathways that were already described to regulate the functional differentiation of mature T lymphocytes (Ribot et al, 2014; Amsen et al, 2015; Duval et al, 2015). Based on their putative binding sites, Map3k2 and Notch2 were thus selected as potential miR-181a(-5p and -2-3p) targets.

To experimentally validate these bioinformatic predictions, γδ thymocytes were transduced with either the empty or the miR-181a viral vector and then differentiated in the presence of IL-2. We next sorted the GFP+ transduced fraction and analyzed the expression of Map3k2, Notch2, and Ptbp1 (as negative control) by RT-PCR. As predicted, miR-181a overexpression in the type 1 differentiation protocol induced a significant decrease in Map3k2 and Notch2 mRNA levels (Fig 4A and B). We also analyzed the expression of genes involved in both MAPK (namely Atf2 and c-Fos) and NOTCH (namely Hes1) signaling pathways and observed a reduction of the expression of all these signature genes within γδ T cells transduced with miR-181a, when compared to the empty control vector (Fig 4B). We further noticed a reduction of additional genes associated with both pathways, namely, p38 and Rbpj (Fig 4B).

Given the described role for both these genes as key regulators of DNA methylation and histone acetylation processes (Clark et al, 2003; Schmeck et al, 2005; Giaimo et al, 2017; Rozenberg et al, 2018), such results point to an alternative upstream transcriptional regulation or epigenetic alterations that can potentially be modulated by miR-181a upregulation. To assess direct binding between miR-181a and its target mRNAs, Map3k2, and Notch2, we designed reporter constructs in a pmirGLO Dual-Luciferase to include the Map3k2 or Notch2 3’ UTR target regions for both miR-181a-5p and -2-3p species (Fig 4A). However, due to the technical limitations to include the entire region of interest from the 3’ UTR Notch2 candidate, we decided to amplify the binding site with the higher percentile context score, as predicted by the TargetScan v8.0 (Fig 4A). We included an additional negative control vector containing a 3’ UTR without miR-181a binding sites. We transiently transfected these constructs into HEK293T cells together with a vector plasmid for either miR-181a or an empty control. Importantly, co-transfection of miR-181a could elicit a significant repression of the luciferase activity of both Map3k2 and Notch2 mRNA targets, while not affecting the luciferase activity of the control mRNA vector (Fig 4C). By contrast, no repression was observed for the candidates or the control mRNAs in cells co-transfected with the empty control. Of note, based on the target score obtained from our *in silico* analysis (Dataset EV1), we also assessed other promising candidate genes, namely, Ifn4 and Stat1,
Figure 3. miR-181a overexpression impairs γδ T cell functional differentiation.

A Retroviral transduction workflow for γδ thymocytes.
B Gating strategy for identification of the GFP+ versus GFP− cells.
C RT–PCR analysis of the expression of miR-181a (−5p and −2–3p) (normalized to the values obtained with the empty virus) in transduced (GFP+) and untransduced (GFP−) γδ thymocytes, cultured with IL-7 plus IL-2 for 11 days (n = 4 independent biological samples).
D FACS analysis of the expression of Annexin V in miR-181a versus empty transduced (GFP+) and untransduced (GFP−) γδ thymocytes, cultured with IL-7 plus IL-2 for 11 days (n = 5 independent biological samples).
E RT–PCR analysis of the expression of Bcl2 and Bcl-xL (normalized to the values obtained with the empty virus) in transduced (GFP+) γδ thymocytes, cultured with IL-7 plus IL-2 for 11 days (n = 5 independent biological samples).
F FACS-derived expression of indicated surface and intracellular markers in miR-181a versus empty transduced (GFP+) and untransduced (GFP−) γδ T thymocytes, cultured with IL-7 plus IL-2 for 11 days (n = 7–11 independent biological samples).

Data information: (C–F) Error bars represent the mean ± SEM. Statistical analysis was performed using the paired Student’s t-test. ns, not significant. *P < 0.05 and **P < 0.01. All experiments were performed with two technical replicates.

Source data are available online for this figure.
two transcription factors crucial for T cell differentiation (O’Shea et al., 2011; Huber & Lohoff, 2014). While the expression of these genes was downregulated in miR-181a transduced cdT cells (Fig 4B), neither Irf4 nor Stat1 were validated as miR-181a targets when first tested in luciferase assays (Fig 4C).

We performed additional luciferase assays by amplifying the 3' UTR regions of Map3k2 and Notch2 in both miR-181a-5p and -2-3p potential binding sites, and included a control condition where the amplified 3' UTR region was mutated in both binding sites. This set of experiments allowed to validate that miR-181a directly
targets Map3k2 and Notch2 (Fig 4C). Here, it is important to highlight the fact that having amplified a 3’ UTR sequence containing a potential binding site for both miR-181a-5p and -2-3p species makes it impossible to clearly define which, or if both strands, regulate Map3k2 and Notch2 mRNA expression. Analogously, the simultaneous mutation of the predicted binding sites for both this miR strands leading to a complete derepression, does not provide a strict answer, and individual luciferase assays where only one of the 3’ UTR predicted (-5p or -2-3p) binding sites is mutated would be required to address this question. This notwithstanding, we correlated the levels of expression of both miR-181a-5p and -2-3p with Map3k2 and Notch2 in γδ thymocytes cultured with IL-7 versus IL-2, and could clearly find an inverse correlation for both miR-181a-5p and -2-3p species (Fig 4D). Of note, only the correlations for the miR-181a-5p species reached a statistical significance. These data, together with the observation of a predominance of the 5p strand in our samples, suggest that Map3k2 and Notch2 are more likely repressed by miR-181a-5p in a physiological context.

Finally, we performed siRNA targeting Notch2 and Map3k2 and achieved a 20–30% reduction in the expression of these genes (Fig 4E). This strategy phenocopied the effect of miR-181a overexpression on NKGD2 expression in peripheral blood γδ T cells, at both the mRNA (Fig 4F) and protein (Fig 4G) levels. Altogether, these data identified Notch2 and Map3k2, two known mediators of T cell differentiation and cytotoxicity (Su et al, 2001; Amsen et al, 2007; Maekawa et al, 2008; Chang et al, 2011), as direct and relevant targets of miR-181a in human γδ T cells. Interestingly, Map3k2 has been shown to negatively regulate TGF-β-mediated T cell differentiation, as Map3k2−/− mice showed an abnormal accumulation of Treg cells in the periphery, with a subsequent decrease in IFN-γ production (Chang et al, 2011). Additionally, genetic loss of Map3k2 in vitro abrogated IL-2 production in T cells (Su et al, 2001). Likewise, Notch2 is well known to modulate T cell activation and differentiation. CD8+ T cells from Notch2-deficient mice display impaired cytotoxic properties caused by the inability to form a transcriptional coactivator complex on the promoter of the gene encoding granzyme B (Maekawa et al, 2008). Notch signaling also contributes to the generation of human γδ thymocytes (Van Coppenolle et al, 2012) and regulates peripheral γδ T cell type 1 effector function and cytotoxic activity (Gogoi et al, 2014). Of note, the miR-181 family has been previously described to have a key role in modulating Notch signaling, as deletion of miR-181a-1/b-1 inhibits the development of Notch1 oncogene-driven T cell acute lymphoblastic leukemia (Fragoso et al, 2012).

In sum, we propose that miR-181a acts as a brake during γδ T cell development, preventing their functional differentiation in the human thymus. This could help explain why contrary to murine γδ T cells, which commit to effector functions in the thymus (Ribot et al, 2009), human γδ thymocytes remain functionally immature (Ribot et al, 2014).

The miR-181 family encompasses six miRs, organized in three clusters: miR-181a/b-1, miR-181a/b-2, and miR-181c/d (Zietara et al, 2013). Importantly, the mature 5p strands for miR-181a-1 and a-2 as well as b-1 and b-2 display identical sequences (Winter & Krueger, 2019), indicating that these miRs might share some common regulatory functions. Within this family, miR-181a/b-1 is the most extensively studied, probably due to its dynamic expression throughout thymocyte development (Winter & Krueger, 2019). In mice, miR-181a expression was shown to be higher in immature DP thymocytes (Neillson et al, 2007; Kirigin et al, 2012), but lower in more differentiated T cell populations, such as Th1 and Th2 effector cells (Li et al, 2007). Mice deficient in miR-181a/b-1 displayed a defect in thymic agonist selection (Zietara et al, 2013; Blume et al, 2016), impacting on invariant natural killer T (iNKT) cell and mucosal-associated invariant T (MAIT) cell development (Zietara et al, 2013; Winter et al, 2019), while retaining normal numbers of γδ T cells (Sandrock et al, 2015).

miR-181a is also known to regulate T cell functions in the context of graft-versus-host disease, where mice receiving donor T cells with enhanced miR-181a expression remained healthy, whereas T cells lacking miR-181a/b-1 induced an exacerbated disease (Lee et al, 2016). Overexpression of different members from the miR-181a family has been previously associated with an impairment of type 1 cytokine production in other immune partners. For instance, previous studies reported a decrease in IFN-γ production upon overexpression of miR-181a-5p in murine CD8+ and human CD4+ T cells (Sang et al, 2015; Amado et al, 2020).

Several studies have highlighted the need to investigate the 3p strands, especially in a pathological context (Jazdzewski et al, 2009; Mitra et al, 2015, 2020; Pink et al, 2015; Misono et al, 2018). In fact, a recent pan-cancer analysis has revealed a miR cooperativity of both (5p and 3p) strands to be able to regulate tumorigenesis and patient survival (Mitra et al, 2020). Here, we used specific primers to assess both miR-181a-5p and -2-3p expression in different γδ T cell samples and observed a consistent higher expression of the -5p compared to the -2-3p strand. Of note, a recent elegant study in mouse shows that miRNA activity is dependent on its concentration and thus suggests that miRNA signatures should incorporate abundance thresholds to establish their regulatory relevance (Rose et al, 2021).

Consistently, based on a mathematical model-derived predictions to describe gene regulation on the single cell level, another pioneer study had previously described that low expressed miRNA below median have low or no inhibitory capability, while miRNAs over the median and third quartile show a range of behavior that does not reflect their expression levels (Lemus Diaz et al, 2017). Of note, the authors propose that the gene regulatory function is actually a dynamic and complex process, in which some miRNAs also display their functionality according to the subcellular location, with low functional and high expressed miRNA being at least partially located in the nucleus, while some mid expressed but high functional miRNAs are mainly seen in the cytoplasmic region (Lemus Diaz et al, 2017).

Albeit the fact that miR-181a-5p was by far the most predominant species present in our study, as the variation in expression of both miR-181a-5p and -2-3p was consistent among the γδ T cells, we postulated that both species could work together to regulate γδ T cell differentiation, and we thus overexpressed both upon transduction of the complete miR-181a hairpin. We show that miR-181a overexpression impairs human γδ T cell differentiation into TNF-α+ effectors, while also reducing the expression of NKGD2. By contrast, we show that miR-181a seemingly did not affect differentiation into IFN-γ producers. This highlights the complexity of action of miRNAs, including the members from the miR-181a family, in fine tuning a restricted gene expression profile by acting on specific

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Conversely, we found the immunosuppressive cytokine, TGF-β, to be the main inducer of miR-181a expression. The concept of miR-181a as a brake to functional γδ T cell differentiation is supported by the fact that its two main cytokine drivers, IL-2 and IL-115, downregulate miR-181a expression. Conversely, we found the immunosuppressive cytokine, TGF-β, to be the main inducer of miR-181a expression in human γδ T cells. We thus propose that the upregulation of miR-181a expression by TGF-β may be part of a negative feedback loop that would prevent excessive inflammation in order to limit tissue damage (Li et al., 2017). On the other hand, established cancer settings are often rich in TGF-β (Batlle & Massagué, 2019), and therefore, we speculate that miR-181a upregulation may trigger γδ T cell functional exhaustion in prostate cancer, as previously reported for other miRs in human T cells in the context of breast cancer (Zhao et al., 2016) or non-small-cell lung cancer (Wan et al., 2018). Interestingly, previous studies demonstrated that tumor-derived exosomal miRs can alter T cell activation or effector profile, thus highlighting potential tumor evasion mechanisms (Ye et al., 2014; Bland et al., 2018).

While miR-181a can impact both NKG2D expression and type 1 cytokine production in differentiating γδ thymocytes, it can no longer regulate the latter process in γδ T cells isolated from the peripheral blood, which are mostly mature (Gibbons et al., 2009; DeBarros et al., 2011; Ribot et al., 2014), with particular effector/memory phenotypes (Deli et al., 2003). This suggests that miR-181a controls type 1 cytokine production at early stages and within a limited time window of γδ T cell differentiation and further implies that γδ T cell maturation status, which presumably associates with dynamic repertoires of mRNA targets, dictates sensitivity to miR-181a. The inverse correlation between miR-181a(-5p and -2-3p) expression and NKG2D levels was found in both differentiating γδ thymocytes and in circulating γδ T cells; and reduced NKG2D expression associated with high miR-181a(-5p and -2-3p) levels in γδ T cells isolated from the blood of patients with prostate cancer. NKG2D plays a critical role in tumor surveillance, by promoting target recognition and cytotoxicity, and thus is particularly relevant to prevent tumor cell metastasis (Sivori et al., 2019). Furthermore, the NKG2D/NKG2DL pathway is being targeted for cancer treatment, and an improved understanding of its post-transcriptional regulation may open new avenues for next-generation immunotherapies.

Concluding remarks

Our study constitutes an important addition to the molecular mechanisms that govern the acquisition of type 1 effector functions of human γδ T cells, and especially at the post-transcriptional level which is less explored. We propose a two-step model for a dynamic miR-181a-based regulation of γδ T cell differentiation, which is finely tuned within pro- versus anti-inflammatory environments. First, in the thymus, elevated concentrations of TGF-β (Juberg et al., 2015) would induce high levels of miR-181a, thus impairing Map3k2 and Notch2 expression, which ultimately would help restrain γδ T cell differentiation. Then, at peripheral inflamed sites, IL-2 or IL-15 signals would reduce γδ T cell expression of miR-181a, thus releasing the functional brake to promote type 1 cytokine and NKG2D expression. This could potentially be mediated by an increase in mir-181a mRNA targets expression, such as Map3k2 or Notch2, combined with other key regulatory mechanisms, such as epigenetic modifications. However, high miR-181a expression levels can be induced de novo in peripheral γδ T cells by TGF-β, which leads to reduced expression of NKG2D as a possible mean to prevent collateral tissue damage. We postulate that tumors take advantage of this mechanism to settle their immune evasion. Thus, we believe our work has pathophysiological implications in cancer immunity and inflammatory disease settings. Importantly, miR-based therapies already exist or are currently under clinical trials, as it is the case for the thyroid cancer combined therapy ThyraMiR®/ThyGeNEXT®, or the Miravirsen treatment against hepatitis C (Lanford et al., 2009; Bonneau et al., 2019). This clearly highlights the potential of using miR-based strategies in the design of new (or improved) γδ T cell-based clinical protocols, toward their effective application in cancer treatment.

Materials and Methods

Patients and healthy donor samples

12 ml of peripheral blood was collected at baseline (before starting new line of therapy) from 119 patients diagnosed with stage IV breast or prostate cancer (breast cancer patients n = 83, 52.3 ± 11.9 years old; prostate cancer patients n = 36, 70.9 ± 8.5 years old) and from 47 healthy donors (female healthy donors n = 27, 46.3 ± 11.3 years old; male healthy donors n = 20, 49.0 ± 8.9 years old), followed at CHULN-Hospital Santa Maria, Lisbon. Thymic specimens were routinely obtained during pediatric corrective cardiac surgery (from new-born to 10-year-old children), performed at Hospital de Santa Cruz Carnaxide. For RT–PCR analysis of miR expression, 15 thymic samples were collected from female (n = 7) and male (n = 8) donors. For RT–PCR analysis of miR expression, 11 thymic samples were collected from female (n = 7) and male (n = 6) donors. Concentrated leukocyte suspension samples (buffy-coats) from healthy donors were collected at Instituto Português do Sangue e Transplantação. For RT–PCR analysis of miR expression, 13 buffy-coat samples were collected from female (n = 5) and male (n = 8) donors. For RT–PCR transduction, 6 buffy-coat samples were collected from female (n = 3) and male (n = 2) donors. For RT–PCR transfection experiments, 6 buffy-coat samples were collected from female (n = 4) and male (n = 2) donors. All samples were collected with informed consent and approved by the Ethical Board of the Academic Medical Center of Lisbon CAML (CHLN/FMUL/IMM). Experiments are conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

Lymphocyte preparations

Blood samples were separated on a Histopaque-1077 density gradient. Thythic samples were cut into small pieces (< 0.5 cm²) and smashed into a 70-µm filter and Histopaque-1077 (Sigma-Aldrich) density gradient. γδ T cells were isolated by magnetic cell sorting (MACS) by positive selection, according to manufacturer’s instructions (Miltenyi Biotec).
Cell culture

MACS isolated γδ T cells were cultured at a density of 10⁶ cells/ml in round-bottom 96-well plates with RPMI 1640 and 2 mM L-glutamine supplemented with 10% FBS, 1% of sodium pyruvate, 1% of HEPES, 1% of minimum essential amino acids (NEAA), and 1% of penicillin and streptomycin (Pen/Strep; all from Invitrogen Life Technologies).

For the analysis of miR-181a expression, γδ T cells were cultured with the following cytokines: IL-2, IL-7, IL-12, IL-15, TNF-α, IFN-γ, IL-4, IL-10, and TGF-β (all from PeproTech, 10 ng/ml). Anti-TGF-β blocking antibody (1D11.16.8, BioXCell, 10 μg/ml) was added when mentioned. γδ T cells from buffy-coats or thymic biopsies were cultured for 4–6 or 11 days, respectively, and then pelleted, frozen, and stored for prior analysis.

For retroviral transduction experiments, γδ T cells were cultured at a 5 × 10⁵ cell/ml density, round-bottom 96-well plates with RPMI 1640 and 2 mM L-glutamine supplemented with 10% FBS, 1% of sodium pyruvate, 1% of HEPES, 1% of minimum essential amino acids (NEAA), and 1% of penicillin and streptomycin (Pen/Strep; all from Invitrogen Life Technologies). On the transduction days, cells were transferred into flat-bottom 96-well plates in a DMEM medium (Invitrogen Life Technologies) supplemented with 15% FBS, 1% of HEPES, 1% of NEAA, and 1% of Pen/Strep. Indicated cytokines were added when mentioned (all from PeproTech, 10 ng/ml).

For retrovirus generation, the human embryonic kidney 293 cells with large T antigen (HEK293T) cell line (ATCC® CRL-11268™) and the NIH/3T3 cells (ATCC® CRL-11268™) were grown in T 175 flasks in DMEM supplemented with 15% FBS, 1% of HEPES, 1% of NEAA, and 1% of Pen/Strep, during 4–5 days. After reaching the required cell number for the experiment, the HEK293T cell line was cultured in 10 cm tissue culture plates (Sigma-Aldrich TPP™) at a density of 5 × 10⁵ cells/ml, while the NIH/3T3 cells were cultured in flat-bottom six-well plates (Corning Incorporated) at a density of 7 × 10⁴ cells/ml.

For dual luciferase assays, HEK293T cell line was cultured in flat-bottom 12-well plates at a density of 1.5 × 10⁵ cells/ml, in DMEM supplemented with 15% FBS, 1% of HEPES, 1% of NEAA, and 1% of Pen/Strep.

For siRNA targeting, γδ T cells were cultured at a 5 × 10⁵ cell/ml density in round-bottom 96-well plates with RPMI 1640 and 2 mM L-glutamine supplemented with 10% FBS, 1% of sodium pyruvate, 1% of HEPES, 1% of minimum essential amino acids (NEAA), without antibiotics. Indicated cytokines were added when mentioned (all from PeproTech, 10 ng/ml).

All incubations were performed at 37°C in a 5% CO₂ environment.

Retroviral transduction of miR-181a

The native precursor stem-loop of the hsa-miR-181a was cloned into the MSCV-IREs-GFP vector (Addgene #9044), modified to include a PGK-GFP-WPRE sequence (pMIG-PGW) as previously described (Schmolka et al., 2018), using human genomic DNA as a template and the following primers for hsa-miR-181a: forward primer, CGCCCGAATTAGATCTCTTACTGATATAGCAGATCCCCAAT and reverse primer, AACCTCGAGAGATCGACTGCTCCTTACCTTGTTGAA. Purified PCR product was then transformed in the Stellar™ Competent Cells according to the manufacturer’s protocol PTS055-2 (Clontech, 2011). The plasmid pMIG-PGW either empty (pMIG-PGW-Empty) or containing the miR-181a stem-loop fragment was extracted from the bacteria using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). The presence of the miR insert was confirmed by DNA digestion using proper restriction enzymes and analyzing the fragment lengths in an agarose gel. Validation of the results was performed by analyzing the sequences, expected and obtained, with the SnapGene® Viewer Software V. 3.1.4. The viral supernatant was produced by transfecting the plasmids pMIG-PGW (either empty or with miR-181a), pCMV-VSV-G and pCCL-Eco with the Opti-MEM® (Life Technologies) and the X-tremeGENE9 DNA Transfection Reagent (Roche) into the HEK293T TAT cells cultured in 10 cm culture plates (x-TremeGene, 2013). Titers of infectious virus were determined by transducing 100,000 NIH/3T3 cells per well in a Nunc™ six-well plate, with 8 μg/ml of polybrene during a 60 min centrifugation at 32°C, 974 g. Cells were infected at a multiplicity of infection of 2.5 or 5, which resulted in a transduction efficiency between 65–80 and 85–98%, respectively.

The pMIG-PGW vector encodes a green fluorescent protein (GFP) that allows to assess the transduction efficiency.

For retroviral transduction of γδ T cells, the latter were cultured in 96-well plate and stimulated overnight with plate-bound mAbs anti-CD3 (1 μg/ml; HIT3a; ebioScience) and anti-CD28 (1 μg/ml; CD28.2; ebioScience) in a media supplemented with IL-2 and IL-7 (10 ng/m PeproTech), for 11 days. 5 × 10⁵ γδ T cells per well were transduced with pMIG-PGW-miR-181a or a pMIG-PGW-Empty vector. The transduction was performed with 4 μg/ml of polybrene (Sigma) followed by a 45 min centrifugation at 32°C, 974 g. One week after the last transduction, γδ T cells were stained with mAbs for FACS analysis. Alternatively, γδ T cells were electronically sorted, based on their GFP expression, in order to assess miR-181a transduction efficiency by RT–PCR.

siRNA gene silencing of Map3k2 and Notch2

γδ T cells were cultured in 96-well plate as previously described and stimulated overnight with plate-bound mAbs anti-CD3 (1 μg/ml; HIT3a; ebioScience) and anti-CD28 (1 μg/ml; CD28.2; ebioScience) in a media supplemented with IL-7 (10 ng/m PeproTech). siRNAs were pre-incubated with the DharmaFECT™ transfection reagent for 20 min, according to the manufacturer’s protocol (Dharmacon). Cells were then transfected with either an siRNA against Map3k2, Notch2, or a negative control siRNA (nontargeting) and analyzed after 48 h. siRNAs were obtained from Dharmacon (ON-TARGETplus Human) and used at a concentration of 500 nM.

Flow cytometry (FACS) analysis

For intracellular cytokine staining, cells were stimulated with PMA (50 ng/ml, Sigma-Aldrich; P-8139) and Ionomycin (1 μg/ml, Sigma-Aldrich; I-0634) for 3 h at 37°C, with the addition of Brefeldin A (BFA; 10 μg/ml, Sigma-Aldrich; B-7651). Cells were stained for the identified cell surface markers for a period of 30 min at 4°C, fixed with Fixation/Permeabilization buffer (ebioScience) supplemented with BFA and left to incubate for 30 min at 4°C. Alternatively, to ensure the maintenance of GFP expression, retrovirally transduced γδ T cells were fixed with 4% Paraformaldehyde (Sigma-Aldrich).
Cells were then permeabilized with Permeabilization buffer (eBioscience) and resuspended in Fc block (eBioscience) for 10 min at 4°C. For intracellular cell staining, cells were incubated for 30 min at 4°C in 50 μl of Permeabilization buffer containing the indicated antibodies.

The following anti-human fluorescently labeled mAbs were used (Table 1): anti-V61-PE (REAI73; Miltenyi Biotec), anti-Annexin V-APC (VAA-33), anti-IFN-γ-APC (4S.B3) (all from eBioscience), anti-TNF-α-PE-Cy7 (Mab11), anti-NKG2D-BV421 (1D11), anti-V62-PerCP (B6), anti-IL-2-BV510 (M1Q-17H12), anti-CD45RA-BV510 (HI100), and anti-CD27-BV605 (O323; all from Biolegend). Live cells were identified through a staining with the LIVE/DEAD (B6), anti-IL-2-BV510 (MQ1-17H12), anti-CD45RA-BV510 (HI100), and anti-CD27-BV605 (O323; all from Biolegend). Live cells were sorted on the FACS Fortessa (BD Biosciences) cell analyzer. All data were analyzed using FlowJo X 10.0.7r2 software (Tree Star).

**RNA isolation, cDNA production, and real-time PCR**

For miR and mRNA expression analysis, total RNA was extracted from MACS isolated γδ T cells or electronically sorted γδ T cells by using the mirNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA concentration and purity were determined using the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA concentration and purity were determined using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific).

For miR, reverse transcription was performed with miRCURY LNA™ Universal RT Kit (Qiagen). For mRNA, reverse transcription from total RNA was performed with random oligonucleotides (Invitrogen) using Moloney murine leukemia virus reverse transcriptase (Promega). Total RNA was reverse-transcribed into cDNA using the T100™ Thermal Cycler (Bio-Rad), and all quantitative PCR (qPCRs) were performed in MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems) using the RT–PCR ViiA7™ system (Applied Biosystems).

For miR expression analysis, LNA™ PCR primer sets (Qiagen) were used on the cDNA previously obtained and relative quantification of specific miRs to small nucleolar RNA C/D Box 44 (SNORD44) reference was carried out using SYBR on ABI ViiA7™ Universal RT Kit (Qiagen). A tenfold dilution series over six points were prepared from the cDNA and used as templates for qPCR. The standard curve was constructed by plotting Cq values against the logarithmic copy numbers of the calibrator oligonucleotides. The amount of an unknown sample was quantified by interpolating the Cq values in the standard curve.

For pre-miR-181a-1-3p and pre-miR-181a-2-3p expression analysis, specific primers were ordered (Eurofins Genomics) to allow for the amplification of each respective stem-loop region, as follows: pre-miR-181a-1-3p forward primer, GACACTCAACCTGCTGCCAGT; reverse primer, GTTACCGATGGTACACATCCAGA; pre-miR-181a-2-3p forward primer, TACAGCAGGCTCCAGCAGG; reverse primer, GTACGCTACCGGTCCAGTCTCTGT. Relative quantification of specific cDNA species was performed as explained above.

Data were analyzed using ViIA7™ software v1.2.1.

**Dual luciferase reporter assay**

The 3’ UTR of Map3k2, Notch2, Irf4, Stat1, and a negative control (Ptbp1) were cloned into pmirGLO vector (Promega) using human genomic DNA as a template and the following primers: Map3k2 forward primer, GCTCGTACGCTGAGTCCCTCCACGATCCCTGCATGGACTGTA; Notch2 forward primer, GCTCGTACGCTGAGTCCCTCCACGATCCCTGCATGGACTGTA; reverse primer, CGACTCTAGACTGAGTCCCTCCACGATCCCTGCATGGACTGTA; negative control, CGACTCTAGACTGAGTCCCTCCACGATCCCTGCATGGACTGTA.

After 48h, firefly and Renilla luciferase activity were measured by using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase activity served as the internal control and results were expressed as relative luciferase activity, normalized to the empty vector.

| Specificity | Clone | Fluorochrome | Cat # | Origin |
|-------------|-------|--------------|-------|--------|
| V61         | REAI73| PE           | 130-120-440 | Miltenyi Biotec |
| Annexin V   | VAA-33| APC          | 88-8007-74 | eBioscience |
| IFN-γ       | 4S.B3 | APC          | 502512   | eBioscience |
| TNF-α       | MAB61 | PE-Cy7       | 502590   | Biolegend |
| NKG2D       | 1D11  | BV421        | 320822   | Biolegend |
| V62         | B6    | PerCP        | 331410   | Biolegend |
| IL-2        | MQI-17H12 | BV510   | 500338   | Biolegend |
| CD45RA      | HI100 | BV510        | 304142   | Biolegend |
| CD27        | O323  | BV605        | 302830   | Biolegend |
Statistical analysis

Statistical analysis and data presentation were performed using GraphPad Prism 8 software (La Jolla). The values presented are mean ± SEM of an independent experiments. The statistical significance of differences between two populations was assessed using either the two-tailed nonparametric Mann–Whitney U test or the Student’s t-test as indicated in the figure legends. The Pearson’s correlation coefficient (r) was used to measure the strength of association between two variables. Correlation Strength (r): 0.1–0.3 weak; 0.3–0.5 moderate; 0.5–1.0 strong. Outlier analysis was applied using the Grubbs’ test (Alpha = 0.01) and the ROUT test (Q = 1%). Statistical significance was indicated at *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Data availability

This study includes no data deposited in external repositories.

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Author contributions

GG designed and performed most of the experiments, analyzed the data, and contributed to the manuscript writing; SC-P assisted in the experiments; PC and PA processed the healthy donor and patient blood samples; LC provided the healthy donors and patients samples; AOG assisted in the experimental design and provided key intellectual input; BS-S provided critical research tools and key intellectual input, and contributed to the manuscript writing; JCR designed the study, supervised the research, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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