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Enhancing adoptive T cell immunotherapy with microRNA therapeutics

Yun Ji^{1,*}, James D. Hocker¹, and Luca Gattinoni^{1,*}

¹Experimental Transplantation and Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, 10 Center Drive, Bethesda, MD, USA

Abstract

Adoptive T cell-based immunotherapies can mediate complete and durable regressions in patients with advanced cancer, but current response rates remain inadequate. Maneuvers to improve the fitness and antitumor efficacy of transferred T cells have been under extensive exploration in the field. Small non-coding microRNAs have emerged as critical modulators of immune system homeostasis and T cell immunity. Here, we summarize recent advances in our understanding of the role of microRNAs in regulating T cell activation, differentiation, and function. We also discuss how microRNA therapeutics could be employed to fine-tune T cell receptor signaling and enhance T cell persistence and effector functions, paving the way for the next generation of adoptive immunotherapies.

Keywords

microRNA (miRNA); tumor immunotherapy; adoptive cell transfer; T cell differentiation, CD8⁺ T cells

Introduction

Adoptive transfer of naturally occurring or genetically redirected tumor-reactive T cells has emerged as one of the most successful immunotherapeutic treatments for patients with advanced hematological malignancies and solid cancers [1, 2]. This therapeutic modality can result in complete and durable responses in a significant fraction of patients with metastases refractory to conventional treatments, but the overall response rate remains unsatisfactory. Advances in our understanding of the biological mechanisms behind the effectiveness of adoptive T cell therapy have underscored the importance of the qualities of transferred T cells [3] and revealed the complexity of the inhibitory barriers posed by the host and tumor cells that need to be overcome for the success of the treatment [4, 5]. Among T cell factors, the avidity of the T cell receptor (TCR) or chimeric antigen receptor (CAR) [6, 7], the proliferative and survival capacities [8–11], and the ability to sustain effector functions

*To whom correspondence should be addressed: jiyun@mail.nih.gov, or gattinol@mail.nih.gov.

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within the tumor [12] have been shown to be crucial determinants for triggering the eradication of malignant cells.

Extensive work has been done to develop new strategies to improve these diverse aspects of the T cell infusion product. For instance, *in vitro* mutagenesis and selection of affinity-matured TCRs or antibodies have been employed to generate high-affinity TCR and CAR [13, 14]. Alternatively, HLA-mismatched donors [15], HLA-transgenic mice [16] and more recently transgenic mice carrying human TCR $\alpha\beta$ gene loci and HLA-A2 [17] have also been used to circumvent tolerance and isolate high-avidity TCRs. Gene engineering approaches to overexpress cytokines [18–20], co-stimulatory molecules [21], anti-apoptotic proteins [22, 23] and cytotoxic molecules [24] have been employed to enhance proliferation, survival and effector functions of adoptively transferred T cells. Because these properties are tightly linked with the maturation state of T cells, there has been an increased interest in developing novel approaches to alter T cell differentiation. These maneuvers include the modification of the cytokine milieu used for *in vitro* cell expansion [25, 26], the manipulation of T cell transcriptional programs [27, 28] and the modulation of T cell metabolism [29–31].

MicroRNA (miRNA) are 21–23 base pair long non-coding RNAs, which mediate post-transcriptional gene silencing [32]. There is now mounting evidence demonstrating that miRNAs are critical players in regulating a wide range of cellular processes including cell proliferation, differentiation, apoptosis, and metabolism [33]. Dysregulation of miRNA expression and activity has been associated with malignant transformation and metastatic behaviors [34]. The past few years have witnessed an explosion of studies aiming at harnessing miRNAs for the treatment of patients with cancer [35, 36]. A largely tumor cell-centric view has led to the development of miRNA therapeutics designed to either block the function of oncogenic miRNAs or to upregulate the expression of tumor-suppressive miRNAs [35, 36]. Here, we propose an entirely different miRNA-based approach for cancer therapy. After summarizing basic aspects of miRNA biology and describing the role of miRNAs in T cell biology, we will discuss how miRNA therapeutics could be employed to enhance the anti-tumor efficacy of adoptively transferred tumor-specific T cells.

miRNA biogenesis and function

MiRNA genes are located in intronic, exonic, or untranslated regions and encoded together with host genes. They are first transcribed by RNA polymerase II into 500–3000 nucleotide pri-miRNAs containing one or multiple stem-loop sequences, and subsequently cleaved by the Drosha-DGCR8 complex to form a 60–100 nucleotide double-stranded pre-miRNA hairpin [37–39]. Pre-miRNAs are then exported into the cytoplasm by Ran GTPase and Exportin 5 and further processed into an imperfect 22-mer miRNA:miRNA duplex by the Dicer protein complex [39, 40]. One of the strands from this duplex – the mature miRNA – binds to Argonaute (AGO) and is incorporated into the RNA-induced silencing complex (RISC) to repress target gene expression [32] (Fig. 1).

Target identification and inhibition is directed by the miRNA ‘seed’ sequence, which is comprised of nucleotides spanning from position 2 to 7 and forms a perfect or near-perfect complementary pair with a 6–8 bp-long motif located within the 3’UTR of target mRNAs

[32, 39]. Once miRNA identifies and binds to the target 3'UTR, the associated 'miRISC' complex initiates mRNA degradation by deadenylation, 5-terminal cap removal and direct exonucleolytic cleavage [32]. The miRISC complex can also block protein translation by interfering with 5'cap recognition and 40S and 60S ribosomal subunit recruitment and assembly, resulting in defective formation of the 80S ribosomal complex [41]. Therefore, miRNAs restrain complementary targets at both mRNA and protein levels.

Although their inhibitory effects on individual proteins are subtle – usually less than 2-fold – miRNAs are potent cellular modulators due to their ability to target multiple molecules within a particular pathway or diverse proteins in converging pathways or biological processes [42]. Furthermore, a single protein can be influenced by several individual miRNAs targeting shared or distinct sequences within the target 3'UTR [42]. Thus, miRNA can potentially regulate biological networks by cumulatively or cooperatively inhibiting consonant molecules. Conversely, they may fine-tune particular signaling pathways by targeting positive and negative regulatory components. Altogether, these features make miRNA a robust and essential arm for coordinated and precise regulation of cellular processes.

Dynamic changes in miRNA expression upon T cell activation and differentiation

Cumulative evidence has demonstrated that miRNAs play critical roles in the development, differentiation, and function of a variety of immune cells including B and T lymphocytes, dendritic cells, and macrophages [43]. Extensive reports have provided a comprehensive view of the expression and functionality of miRNAs in diverse lymphoid cell lineages [44–47]. Here we will review miRNAs specifically expressed in mature T lymphocytes, with a particular focus on those influencing CD8⁺ T cell differentiation and function.

Upon activation, T cells undergo global gene expression remodeling to support cell growth, proliferation, and effector functions. These transcriptional changes are also accompanied by substantial and rapid modifications of the T cell miRNAome [48]. Following T cell stimulation, acute proteasomal degradation of AGO results in severe miRNA instability and global depletion [49]. A handful of miRNAs, including miR-17, miR-18a, miR-19a/b, miR-20a, miR-21, miR-31, miR-130, miR-146a, miR-155, miR-214 and miR-301, are instead upregulated in response to T cell activation [49–54]. While miR-19b, miR-31, miR-155 and miR-214 are rapidly upregulated, peaking at 12–24h after activation [49, 51], the induction of miR-21, miR-130, miR-301, and miR-146a exhibits slower kinetics, peaking three to six days after initial stimulation [50, 52, 53]. The strength of T cell stimulation appears to dictate the induction levels for some of these miRNAs. For instance, the magnitude of miR-155 expression strongly correlates with TCR affinity [55]. Costimulation is also critical for proper induction of miRNAs as demonstrated by the observation that blockade of CD28 signaling by CTLA4-Ig mitigates miR-214 expression in activated T cells [51]. Thus, miRNA expression dynamically changes upon T cell activation, suggesting that tight regulation of miRNA expression is essential for precise and effective immune responses.

Depending on the strength and quality of stimulatory signals received during priming, naïve T cells differentiate into a variety of memory and effector cell subsets characterized by distinct phenotypic and functional profiles [3, 56, 57]. Despite the considerable wealth of information available regarding the transcriptional [56–58], epigenetic [57, 59] and metabolic changes [60, 61] that accompany T cell differentiation, it is surprising how limited the existing data on miRNA expression in different T cell subsets is. Naïve T cells express the large majority of miRNAs, including miR-15b, miR-16, miR-142-3p and -5p, miR-150, and the let-7 family [62, 63]. These miRNAs are progressively downregulated during T cell differentiation, showing intermediate levels of expression in memory cells and the lowest levels in effector cells [62, 63]. By contrast, a limited number of miRNAs, such as miR-155, miR-21, miR-146a and the miR-17~92 cluster, are highly expressed in effector T cells compared to naïve cells [62–64]. This expression pattern is strikingly conserved in mouse and human CD8⁺ T cells, suggesting that global modulation of miRNA levels during T cell differentiation is a functionally relevant event.

CD8⁺ T cell activation and differentiation must be tightly regulated to induce productive immune responses and establish long-term immunological memory [56, 57]. Alterations in the nature, duration and setting of antigen stimulations can result in dysfunctional T cell states such as anergy, tolerance and exhaustion [65, 66]. Schietinger et al. [67] have recently shed light on miRNAs potentially contributing to CD8⁺ T cell tolerance. Unexpectedly, only three miRNAs – miR-21, miR-184, and miR-181a – were differentially expressed in tolerant cells compared to cells that were functionally rescued under lymphopenic conditions [67]. MiR-181a was highly expressed in tolerant cells, whereas miR-21 and miR-184 were overexpressed in rescued cells, possibly as a result of functional reactivation and subsequent proliferation. Notably, known and predicted targets of miR-181a were reduced in tolerant cells, suggesting a key role for this miRNA in the establishment and maintenance of tolerance [67]. Exploration of the miRNA landscape in mature CD8⁺ T cells has just begun; future studies are warranted to comprehensively dissect miRNA expression across all physiological and dysfunctional T cell states and subsets.

Critical miRNAs regulating T cell effector differentiation

MiRNAs have emerged as pivotal regulators of T cell development and effector function. Evidence stems from the observation that Dicer, a molecule essential for the processing and generation of mature miRNAs, is required for proper thymocyte development, T cell differentiation, and effector cytokine production [50, 68]. In the absence of Dicer, T cell development is compromised resulting in defective numbers of peripheral T lymphocytes [50, 68]. Both CD4⁺ and CD8⁺ T cells deficient of Dicer exhibit altered proliferation and are prone to apoptosis upon TCR stimulation [50, 68]. Moreover, T cells lacking Dicer experience more pronounced activation and preferentially differentiate into short-lived effector cells [50, 68]. For instance, Dicer-deficient CD4⁺ T cells exhibit overt Th1 programming characterized by increased production of IFN- γ [68]. Likewise, CD8⁺ T cells lacking Dicer are hyper-activated following stimulation and display rapid and sustained expression of the activation marker CD69 [50]. Dicer deficient CD8⁺ T cells also express higher levels of perforin and granzyme B and show moderate increases in the secretion of effector cytokines IFN- γ and TNF- α , demonstrating a propensity to differentiate into

cytotoxic effector T cells [69]. Reminiscent of the Dicer knockout phenotype, depletion of miRNAs in AGO2-deficient T cells resulted in enhanced differentiation and effector cytokine production [49].

Findings from studies employing mice with defective miRNA processing machinery have demonstrated that the dominant function of miRNAs in T cells is to restrain their differentiation. Physiologically, global depletion of miRNAs in activated T cells is achieved by multiple mechanisms, including acute proteasomal degradation of AGO [49] and progressive downregulation of Dicer protein in differentiating cells [69]. Recently, key miRNAs responsible for the maintenance of T cell naivety have begun to be revealed. MiR-139 and miR-342 were found to target and inhibit essential components of the transcriptional and cytotoxic machinery of effector cells such as Eomesodermin (EOMES) and perforin [69]. In addition, miR-29 has been shown to repress effector T cell programming by simultaneously targeting non-redundant master regulators of cytotoxic T cell differentiation T-BET and EOMES, as well as IFN- γ [70, 71]. MiR-150 has also been proposed to inhibit the development of effector T cells by limiting the pro-differentiating action of IL-2 through down-regulation of IL-2 receptor α [69]. However, recent findings have revealed that deletion of miR-150 in CD8⁺ T cells impaired effector immune responses, indicating that this miRNA rather promotes T cell proliferation and differentiation [72]. While experiments using Dicer and AGO2-deficient mice have proven to be fundamental, studies focusing on manipulation of individual miRNAs or miRNA clusters provide a deeper understanding of their role in specific biological contexts.

As discussed above, miR-155 is one of the few miRNAs upregulated upon T cell activation [49]. This miRNA is considered an indispensable immuno-miRNA as it is required for appropriate immune responses [73]. Mice lacking B-cell integration cluster (BIC), the gene encoding miR-155, display impaired immune cell development and function that is not restricted to T lymphocytes but also involves other immune components such as B cells, NK cells and dendritic cells [73–75]. A number of recent studies have demonstrated that miR-155 is essential for mounting CD8⁺ T cell effector responses against intracellular pathogens and cancer [55, 76–79]. Impaired effector responses are especially pronounced under conditions in which CD8⁺ T cells face continuous antigenic stimulation such as chronic infections and tumors, leading to uncontrolled viral replication [55, 77, 79] and unrestrained tumor growth [55, 76]. Regardless of the disease model, miR-155-deficient CD8⁺ T cells uniformly showed impaired expansion, poor survival, and decreased production of effector cytokines such as IFN- γ and TNF- α [55, 76–79]. The underlying biology is multifaceted, but it appears to converge on a few signaling pathways that are severely dysregulated in the absence of miR-155. T cells deficient of miR-155 exhibit defective PI3K-AKT signaling [79] due to heightened levels of the inositol 5-phosphatase SHIP-1 [76, 80]. The signal transducer and activator of transcription (STAT) pathways are also influenced by miR-155 activity [55, 77, 81]. For instance, the accumulation of the miR-155 targets Suppressor of Cytokine Signaling 1 (SOCS-1) [55, 81] and the protein tyrosine phosphatase PTPN2 [81] in miR-155 deficient cells dampens STAT5 signaling. Deficiency of miR-155 has also been associated with increased activity of STAT1 and other type I interferon-associated transcription factors such as IRF7 [77], although the biological mechanism underlying this observation has not been determined. Recent findings using a

transgenic mouse model in which the regulation of SOCS-1 by miR-155 is specifically abolished have underscored how miR-155 biology in T cells is largely attributable to the repression of this molecule, especially in settings of chronic antigenic stimulation [82]. The failed physiological suppression of SOCS-1 by miR-155 resulted in severely impaired expansion and survival of viral-specific CD8⁺ T cells following chronic LCMV infection [82]. Consistent with these observations, enforced expression of SOCS-1 recapitulated the miR-155 knockout phenotype [55].

Another series of studies have recently focused on the activity of the polycistronic miR-17~92 cluster, which is also highly expressed in effector CD8⁺ T cells [64]. This cluster encodes six miRNAs – miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92 – which are generated via differential processing from a common pri-miRNA transcript [83]. Conditional deletion of miR-17~92 in viral-specific cells impaired cell proliferation and differentiation, resulting in lower numbers of antigen-specific cells with reduced effector molecule expression [64]. Mechanistically, miR-17~92 enhances mammalian target of rapamycin (mTOR) signaling by targeting upstream inhibitory phosphatases such as Phosphatase and tensin homolog (PTEN) [64, 84] and PH Domain And Leucine Rich Repeat Protein Phosphatase 2 (PHLPP2) [84]. Consistently, miR-17~92 overexpression enhanced mTOR activity in CD8⁺ T cells and promoted the development of terminal effectors at the expense of memory T cells [64]. Notably, genetic manipulation of miR-17~92 levels was associated with opposing changes in the expression of the DNA-binding inhibitor Id3, a key transcriptional regulator of memory differentiation, further supporting the notion that miR-17~92 levels control the balance of effector and memory formation [28, 64, 85, 86].

Similar to miR-155 and the miR-17~92 cluster, miR-146a is also upregulated in T cells following TCR engagement. However, contrary to these miRNAs, miR-146a functions as a negative regulator preventing excessive and unrestrained T cell activity [52]. In the absence of miR-146a, CD8⁺ T cells are hyper-responsive, mounting more potent primary and secondary immune responses [52]. In a model of chronic inflammation, uncontrolled T cell activation resulted in the accumulation of effectors in peripheral tissues and the development of T cell-mediated autoimmunity [52]. MiR-146a was found to act in a negative feedback loop to dampen TCR-induced NF-κB activity by targeting the NF-κB signaling transducers TRAF6 and IRAK1 [52, 87]. Altogether, studies from mice deficient of specific miRNAs strengthen the notion that miRNAs are indispensable players regulating proper T cell immunity.

Harnessing miRNA biology to enhance adoptive T cell immunotherapy

The capacity of miRNAs to target multiple proteins in converging pathways and their involvement in regulating various aspects of T cell biology make them attractive molecules to exploit in the pursuit of more effective adoptive T cell therapies. Here, we describe methods to enhance T cell sensitivity to tumor antigens, fitness, and effector functions by harnessing miRNA biology (Fig. 2).

Enhancing TCR sensitivity

T cell sensitivity to antigen is intrinsically regulated to ensure balance between immunity and tolerance [88]. The increased accessibility of high throughput deep-sequencing technology has recently revived interest in targeting tumor-specific neoantigens, based on the notion that TCR recognizing this class of antigens are highly avid [89, 90]. However, because immunogenic neoantigens typically arise on the basis of individual cancers, therapies that target them must be tailored to individual patients. By contrast, the shared nature of non-mutated, tumor-associated self antigens has made them an attractive target for T cell-based immunotherapy, but intrinsic tolerance mechanisms limit the avidity and effectiveness of the vast majority of T cells targeting this type of antigen [91].

Extensive efforts to enhance antigen reactivity and circumvent tolerance have focused on identifying rare, high avidity self/tumor reactive TCR [90, 91]. Alternatively, modulation of TCR signaling thresholds to enhance T cell activation and function has also been explored [12, 92, 93]. Because several miRNAs have been found to influence TCR signaling by targeting key inhibitory phosphatases [47], they may be promising candidates to manipulate for this purpose. For instance, the tyrosine phosphatase PTPN2, which negatively regulates TCR signaling by repressing LCK Proto-Oncogene, Src Family Tyrosine Kinase (LCK) and FYN Proto-Oncogene, Src Family Tyrosine Kinase (FYN) [94], can be efficiently repressed by miR-155 [81]. Another promising candidate, miR-21, promotes T cell activation in a positive feedback loop by down-regulating the serine/threonine phosphatases DUSP10 [95, 96] and Sprouty 1 [97, 98], which suppress TCR-induced JNK and ERK phosphorylation [98, 99]. Consistently, miR-21 overexpressing T cells have been shown to induce severe autoimmune arthritis upon adoptive transfer [100], supporting the idea that enforced expression of this miRNA can be utilized to boost T cell immunity. The capacity of miR-181a to simultaneously target multiple serine/threonine and tyrosine phosphatases may make it the most attractive candidate in this category. This miRNA has been shown to enhance LCK and ERK activity by inhibiting DUSP5, DUSP6, PTPN11 (also known as SHP2), and PTPN22 [101] and to govern central and peripheral tolerance [102, 103]. Indeed, overexpression of miR-181a in T cells increased TCR sensitivity to cognate antigen and enhanced intracellular calcium flux upon TCR triggering, resulting in more pronounced release of IL-2 [101]. However, the recent observation that miR-181a is enriched in tolerant tumor-reactive T cells [67] warrants in-depth characterization of this miRNA in the tumor immunotherapy setting.

Improving T cell fitness

The ability of T cells to engraft and persist, here referred to as ‘T cell fitness’, is a critical determinant of the success of adoptive T cell therapies [3]. Indeed, T cell persistence has been shown to correlate strongly with the induction of objective tumor responses in numerous clinical studies [10, 11, 104, 105] and preclinical animal models [8, 9, 27, 29–31, 106]. The survival capacity of adoptively transferred T cells is dependent upon both their responsiveness to homeostatic cytokines and their resistance to cell death [107]. To support T cell engraftment and enhance fitness, adoptive immunotherapies are currently performed in conjunction with the provision of exogenous cytokines and lymphodepletion conditioning, which removes endogenous lymphocyte populations competing for access to

limited amounts of homeostatic cytokines [108, 109]. We have recently reported that miR-155 overexpression can be employed to enhance T cell responsiveness to homeostatic γ_c cytokines in lymphoreplete hosts, resulting in enhanced T cell survival and superior antitumor responses [81]. MiR-155 facilitates cytokine signaling by concurrently targeting multiple negative regulators upstream of the PI3K/AKT and STAT pathways [81]. This cell-intrinsic strategy has the potential to reduce the severe toxicities associated with non-specific maneuvers such as lymphodepletion [110] and exogenous cytokine administration [111]. Furthermore, it also provides the possibility to deliver multiple rounds of T cell infusions in short succession, an approach that is currently prohibited by the toxicities associated with preconditioning treatments.

The fitness of transferred cells might be also boosted by engineering T cells to express the miR-17~92 cluster. By suppressing the PI3K/AKT inhibitor PTEN and the pro-apoptotic molecule BIM, miR-17~92 has been shown to support extensive proliferation and accumulation of effector T cells [64, 85]. However, sustained miR-17~92 and AKT activity can also lead to defective memory cell development [64, 112], which may be detrimental to long-lasting anti-tumor responses. Recently Ohno *et al.* have provided evidence of the potential benefit of overexpressing miR-17~92 in tumor-specific T cells [113]. Enforced expression of the miR-17~92 cluster in anti-EGFRvIII CAR-modified T cells provided prolonged protection against tumor re-challenge in a xenograft model of human glioblastoma [113]. Like miR-17~92, miR-214 also targets PTEN [51] and might be harnessed to enhance AKT-driven T cell expansion, although its effect in models of adoptive immunotherapy has yet to be evaluated.

The overexpression of miRNAs that suppress proapoptotic molecules represents another therapeutic opportunity. For example, miR-15b, which has been shown to target and inhibit Death Effector Domain-containing DNA binding protein (DEDD) [114], might be used to enhance the survival of adoptively transferred T cells. However, overexpression of this miRNA can also inhibit T cell activation and lessen IL-2 and IFN- γ secretion, potentially damaging antitumor effector responses [114]. Whether the beneficial effect on T cell fitness exceeds the negative impact on T cell activation remains to be addressed.

Antagonizing miRNAs that negatively regulate T cell immune responses is another theoretically appealing approach to increase T cell fitness and antitumor function. As discussed previously, miR-146a is a major suppressor of NF- κ B signaling that is upregulated in response to T cell activation in order to dampen effector responses [52]. AntagomiRs, miRNA decoys, or miRNA sponges targeting miR-146a could be employed to augment NF- κ B activity in adoptively transferred cells, potentially enhancing the potency of their anti-tumor responses. Because increased expression of several of the miRNA discussed here, including mir17~92, miR-21 and miR-155, has been associated with the development or aggressiveness of malignancies, their overexpression in tumor-specific T cells warrants some precautions. Safety could be ensured by introducing suicide genes [115, 116] or alternatively by employing inducible promoters to tightly control miRNA expression [117].

Augmenting effector functions

To successfully eradicate tumors, adoptively transferred T cells must ultimately migrate into tumor deposits, release substantial amounts of pro-inflammatory cytokines, and exert potent cytotoxicity. Productive effector responses, however, are often impeded by multiple cellular and molecular immunosuppressive hurdles within the tumor microenvironment [4].

Transforming growth factor-beta (TGF- β) is a key immunosuppressive cytokine that profoundly shapes the tumor milieu [118]. There have been extensive efforts to inhibit TGF- β signaling in patients with cancer, but the broad involvement of this signaling pathway in numerous homeostatic processes and the potential adverse effects of such treatments in normal tissues have hampered the development of anti-TGF- β therapies [118].

Adoptive T cell therapy offers the advantage of targeting TGF- β activity in transferred cells without affecting the entire host. Genetic engineering of TGF- β dominant negative receptors in tumor-specific T cells has proven to be an effective approach in preclinical mouse studies [119–121]. As TGF- β signaling is intimately involved with miRNA biogenesis [122], an alternative strategy is to counter the TGF- β -induced program in T cells by artificially modulating miRNA levels. It was recently reported that release of TGF- β within the tumor microenvironment increased miR-23a expression in CD8⁺ T cells, resulting in suppression of its target Blimp1, a master regulator of effector T cell differentiation [123–126].

Accordingly, granzyme B and IFN- γ levels negatively correlated with the amount of miR-23a in T cells [123]. Overexpression of a miR-23a decoy attenuated the immunosuppressive effects of TGF- β , increased granzyme B and IFN- γ expression, and enhanced the antitumor effectiveness of adoptively transferred CD8⁺ T cells in mouse models of established tumors [123]. The miR-17~92 cluster might also be employed to mitigate the inhibitory influence of TGF- β signaling on transferred T cells. By inhibiting the TGF- β type II receptor (TGF- β RII), miR-17~92 overexpression has been shown to promote CD8⁺ T cell proliferation and enhance IFN- γ production and cytotoxicity following cognate antigen stimulation [127]. Finally, enforced expression of miR-21, a key miRNA involved in the TGF- β pathway, might be utilized to overcome TGF- β tolerogenic effects. This miRNA is induced by TGF- β and is thought to act in a negative feedback loop that limits the intensity and duration of TGF- β signaling by down regulating TGF- β RII directly [128]. Interestingly, upregulation of miR-21 was associated with exit from tolerance and functional T cell reactivation [67]. In agreement with these findings, a recent report showed that miR-21 overexpressing T cells displayed enhanced IFN- γ and TNF- α production upon stimulation [129]. Altogether, these studies indicate the potential for miRNA therapeutics to mitigate the effects of TGF- β -mediated immunosuppression on adoptively transferred tumor-reactive T cells.

Several miRNAs involved in silencing molecules that are essential for T cell effector functions have been recently identified, and represent another exciting class of targets to antagonize using miRNA inhibitors. For example, inhibition of miR-139 and miR-342, which downregulate perforin and EOMES [69], could be employed to boost T cell cytotoxicity. In addition, IFN- γ release could be potentiated by targeting miR-29. This miRNA has emerged as a major negative regulator of IFN- γ synthesis that functions both by directly binding to the *Ifng* 3'-UTR and suppressing transcriptional factors that are required

for optimal IFN- γ production, such as T-BET and EOMES [70, 71]. Recently, Ma *et al.* demonstrated that transgenic mice expressing a ‘sponge’ to deplete endogenous miR-29 exhibit high circulating levels of IFN- γ in response to bacterial challenge and enhanced bacterial clearance [69]. Although miR-29 sponges have not been evaluated in the context of tumor-bearing hosts, it is conceivable that suppression of miR-29 activity might lead to improved antitumor immunity.

A potential drawback of this strategy is that the enforcement of the effector program might lead to terminal differentiation and senescence [124–126, 130], thus impairing T cell fitness and anti-tumor responses. Therefore, it would be desirable to engineer miRNA and miRNA inhibitors potentiating effector differentiation under the control of inducible promoters to spatially and temporally regulate their expression. To this end, the NFAT promoter could be employed to boost effector functions within tumor masses, as it will drive miRNA expression only upon cognate antigen stimulation [117].

Concluding remarks

It is now well established that miRNAs are pivotal regulators of T cell activation, proliferation, survival, differentiation, and effector functions, which are all key factors affecting the therapeutic outcome of adoptive immunotherapies. An ever-growing understanding of the role that miRNAs play in these processes is bringing us closer to the prospect of safer and more effective T cell therapies based on miRNA therapeutics. Harnessing miRNAs has multiple advantages over other gene engineering strategies. Due to their ability to target multiple molecules simultaneously, manipulation of a single miRNA can radically change T cell fate and behavior [42]. Modulation of miRNA levels also allows for fine-tuning of cellular processes without the deleterious effects that can result from overexpression of constitutively activated transcription factors or enzymes [112]. Lastly, thanks to their small size, miRNA genes can be easily integrated into existing multicistronic TCR and CAR platforms. In summary, miRNA gene engineering of tumor-specific T cells is a powerful and versatile approach that has the potential to revolutionize the next generation of adoptive T cell-based immunotherapies.

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Highlights

- miRNA expression is dynamically regulated following CD8⁺ T cell activation.
- miRNAs critically regulate CD8⁺ T cell differentiation and function.
- miRNA therapeutics can be used to enhance T cell-based immunotherapies.

Fig. 1. MicroRNA biogenesis

The miRNA gene is transcribed into pri-miRNA by RNA polymerase II (Pol II) within the nucleus and processed into Pre-miRNA by the DROSHA-DGCR8 complex. Pre-miRNA is subsequently transported by Exportin5 and Ran GTPase into the cytoplasm and further processed by the DICER complex into a miRNA:miRNA duplex. Finally, mature miRNA binds to AGO (Argonaute) and is incorporated into the RISC (RNA-induced silencing complex), leading to mRNA degradation and inhibition of protein translation.

Fig. 2. Harnessing miRNA biology to enhance adoptive T cell immunotherapy

miRNA, anti-miRNA (bold font) and their downstream targets could be employed to augment tumor destruction by enhancing T cell receptor (TCR) sensitivity (upper left), T cell fitness (upper right), and effector functions (lower panel). DUSP, Dual Specificity Phosphatase; SPRY1, Sprouty Homolog 1; PTPN, Protein Tyrosine Phosphatase, Non-Receptor Type; JNK, JUN N-Terminal Kinase; DEDD, Death Effector Domain Containing; PTEN, Phosphatase And Tensin Homolog; TRAF6, TNF Receptor-Associated Factor 6; IRAK1, Interleukin-1 Receptor-Associated Kinase 1; SHIP-1, SH2 Domain-Containing Inositol 5-Phosphatase 1; SOCS-1, Suppressor Of Cytokine Signaling 1; PI3K,

Phosphatidylinositol 3-Kinase; STAT5, Signal Transducer And Activator Of Transcription 5; TGF- β , Transforming Growth Factor, Beta; TGF- β RII, Transforming Growth Factor, Beta Receptor II; BLIMP1, B lymphocyte-induced maturation protein-1; EOMES, Eomesodermin; IFN- γ , interferon gamma.

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