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MicroRNAs in Immune Regulation - Opportunities for Cancer Immunotherapy

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Abstract

Endogenously produced microRNAs are predicted to regulate the translation of over two-thirds all human gene transcripts. Certain microRNAs regulate expression of genes that are critically involved in both innate and adaptive immune responses. Immune cells represent a highly attractive target for microRNA gene therapy approaches, as these cells can be isolated, treated and then reintroduced into the patient. In this short review, we discuss how recent discoveries on the roles of microRNAs in immune-regulation will advance the field of cancer immunology and immunotherapy. Targets identified already in T cells include microRNAs, miR-17-92 family, miR-155, and miR-181a. In macrophages, miR-125b, miR-146, and miR-155 act as Pathogen Associated Molecular Pattern Molecule-associated microRNAs and miR-34C and miR-214 as Damage Associated Molecular Pattern Molecules-associated miRs. We have also demonstrated that the ability of tumors to serve as targets for cytolytic effectors is regulated by miR-222 and miR-339.

Keywords

microRNA; Type-1 helper (Th1); cancer; cancer immunology; high-mobility group box (HMGB)1

microRNA

microRNAs (miRs) are endogenous single-stranded RNA molecules which are 18-24 nucleotides in length (Hammond, 2006). Mature miRs repress mRNA encoded protein translation and are highly conserved between species, including viruses, plants and animals (Elmen et al., 2008). There are over 700 miRs that have been identified in the human genome

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(http://www.mirbase.org/). Although transcriptional targets are predicted, most of these have yet to be validated, making this a rich area for investigation.

Genes encoding miRs are transcribed by RNA polymerase II into long primary miR sequences (pri-miRs) with a 5' cap, 3' untranslated region UTR), and a hairpin sequence that encodes the mature miR. The hairpin of the pri-miR is then cleaved by the enzyme Drosha to form precursor miRs (pre-miRs), which are then transported via Exportin V to the cytoplasm. Once in the cytoplasm, the RNase III super family member enzyme Dicer cleaves the loop in the pre-miRs leaving two individual strands of RNA. One strand of the miR is usually degraded and the other strand associates with the RNA induced silencing complex (RISC) and can bind to the 3' UTRs of target mRNAs and inhibit translation. A major determinant in this recognition process is complementarity between the target mRNA 3' UTR and the seed region of 6–8 nucleotides at the 5′ end of the miR.

The usual consequence of miR:mRNA interaction is the down regulation of protein expression by translational repression or mRNA cleavage and decay (Kim, 2005). Complete complementarity of the miR to the mRNA 3' UTR results in mRNA cleavage and subsequent degradation, while partial complementarity of the miR to the 3' UTR sequence inhibits circularization of the mRNA, needed for ribosomal attachment and translational repression (Ying and Lin, 2009). Following these interactions, miRs then regulate crucial cellular processes such as proliferation (Cheng et al., 2005), apoptosis (Xu et al., 2004), development (Karp and Ambros, 2005), differentiation (Chen et al., 2004), metabolism (Poy et al., 2004), and immunity [reviewed in (Sonkoly et al., 2008, Kanellopoulou and Monticelli, 2008, Lindsay, 2008, Tili et al., 2008, Li et al., 2007, Pedersen et al., 2007, Williams et al., 2008, Pauley and Chan, 2008, Asirvatham et al., 2009, Bellon et al., 2009, Xiao and Rajewsky, 2009)].

Dicer and miRs in the Development of T cells

When Dicer, the key enzyme in miR biogenesis, is conditionally inactivated in (Cobb et al., 2005) immature thymocytes [double negative 3 (DN3)] stage, a 10-fold reduction in T cell receptor (TCR)-αβ expressing thymocyte numbers, and drastic reduction in the more mature double-positive ($CD4^+CD8^+$) and single-positive ($CD4^+$ or $CD8^+$) thymocytes are found. These mutant cells exhibit reduced proliferation and increased cell death following activation *in vitro*. When cultured under T helper (Th)1 or Th2 polarizing conditions, they manifest a profound bias toward Th1 differentiation, reflecting the failure of these cells to repress interferon-γ (IFN-γ) expression (Muljo et al., 2005). The T cell subset most affected by the deletion of *Dicer* at the double-positive stage are the T regulatory (Treg) cells, resulting in severe immunopathology developing in the mutant mice, characterized by splenomegaly, enlarged intestinal lymph nodes, and colitis (Cobb et al., 2006). Furthermore, when Dicer is selectively knocked out in FoxP3 expressing T cells, a significant percentage of the Treg lineage cells exhibit a T helper cell memory phenotype including increased levels of CD127, interleukin (IL)-4, and IFN-γ (Zhou et al., 2008). Importantly, Dicer-deficient Treg cells lose suppressive activity *in vivo*, and these mice rapidly developed fatal systemic autoimmune disease (Zhou et al., 2008). These results support a central role for miRs in maintaining differentiated Treg cell function *in vivo* and homeostasis of the adaptive immune system.

The spectrum of miR expression changes during lymphoid and myeloid development, as well as following antigen recognition (Chen et al., 2004, Wu et al., 2007, Georgantas et al., 2007). miR expression profiling of naïve, effector and memory $CD8^+$ T cells revealed the dynamic regulation of miRs during antigen-induced $CD8^+$ T cell differentiation (Wu et al., 2007). In addition, external stimuli, such as cytokines, also lead to a rapid shift in miR levels. For example, miR-125b and miR-155 levels change rapidly in response to lipopolysaccharide or

tumor necrosis factor (TNF) stimulation in a mouse macrophage cell line Raw 264.7 (Tili et al., 2007). We have recently found that IL-4, and signal transducer and activator of transcription (STAT)6 signaling down-regulate miR-17-92 expression in both murine and human T cells (Kohanbash *et al*. submitted). Furthermore, IFN-β rapidly modulates the expression of numerous cellular miRs, which target genomic RNA within the hepatitis C virus and confer antiviral effects (Pedersen et al., 2007). miR-26 expression in hepatoma patients is associated with enhanced survival and response to adjuvant therapy with IFN-α (Ji et al., 2009).

Macrophage miRs in Development

Myeloid cells including monocytes and macrophages regulate tissue homeostasis and local inflammation/immunity, differentiating into various cell types in response to provocative stimuli (Mantovani et al., 2005, Randolph et al., 2002, Chomarat et al., 2003). Understanding differences in response to tissue injury or damage, and in particular stimuli arising from damage or pathogen associated molecular patterns (DAMPs or PAMPs) lead to characteristic miR changes (Figure 1). Alternatively, they can differentiate into antigen presenting cells [dendritic cell (DC)1 or DC2] or myofibroblasts (Randolph et al., 2002, Chomarat et al., 2003) in response to various stimuli. Surface markers expressed on macrophages change significantly in response to either DAMPs or PAMPs, coordinate with their change in function (Pattison et al., 1994, Grau et al., 2000, Lan et al., 1998, Scriba et al., 1998). Gene expression profiles in breast epithelial cells change significantly in response to hypoxia or acidosis (Chen et al., 2008), and we would presume that this also occurs in macrophages and be regulated by miRs. Oxygen can serve as an integral component of the healing cascade in oxygen-dependent redox-senstive signaling processes (Sen and Roy, 2008).

From the rapidly growing body of evidence demonstrating specific regulation of immune function by miRs, in the following sections, we have selected and discussed miRs that appear to have a particularly important role in cancer immunology and immunotherapy, primarily focusing on T cells, myeloid cells (Tili et al., 2009, Liu et al., 2009, Martinez-Nunez et al., 2009, Chen et al., 2009, Androulidaki et al., 2009, Hou et al., 2009, Lu et al., 2009), and tumor cells.

miR-17-92

Many of the known miRs appear in clusters on single polycistronic transcripts (Tanzer and Stadler, 2004, He et al., 2005). The miR-17-92 transcript encoded by mouse chromosome14 (and human chromosome 13) is the precursor for 7 mature miRs (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1). This cluster is homologous to the miR-106a-363 cluster on the X chromosome and the miR-106b-25 cluster on chromosome 5 in the mouse and the syntenic chromosome 7 in human. Together, these three clusters contain 15 miR stem-loops, giving rise to 14 distinct mature miRs that fall into 5 miR families. miRs in the miR-17-92 cluster are amplified in various tumor types, including B-cell lymphoma and lung cancer, and promote proliferation and confer anti-apoptotic function in tumors, thereby promoting tumor-progression (He et al., 2005, Hayashita et al., 2005, Matsubara et al., 2007, Lawrie, 2007, Rinaldi et al., 2007). miR-17-92 has thus been described as an oncogenic miR (thereby an onco-miR), although ectopic expression of miR-17-92 in T cells by itself did not induce cancers (Xiao et al., 2008).

Knockout and transgenic studies of the miR-17-92 cluster in mice demonstrate the importance of this cluster in mammalian biology (Xiao and Rajewsky, 2009). Knockout of the miR-17-92 cluster results in immediate post-natal death. Knockout of miR-106a and miR-106b clusters either individually or together demonstrate no apparent change in phenotype. However, when the miR-17-92 cluster is knocked out together with the miR-106a or 106b cluster, the result is embryonic lethality (Xiao and Rajewsky, 2009).

Several transcription factors have been identified that regulate expression of the miR-17-92 cluster, including members of the E2 transcription factor (E2F) family (Woods et al., 2007, Sylvestre et al., 2007), c-Myc (O'Donnell et al., 2005), STAT3 (Brock et al., 2009), as well as the sonic hedgehog pathway (Northcott et al., 2009, Uziel et al., 2009). In addition, despite miRs in the miR-17-92 cluster are derived from a single polycistronic transcript, different levels of expression among the families within the miR-17-92 cluster were observed in a variety of lymphocytes at various developmental stages (Xiao et al., 2008). This could be due to different efficiencies of processing for generation of mature miR and/or different stability levels of mature miRs. Further studies are warranted to explain these events and find possible implications for near future therapeutic translation of miR biology.

Genes targeted by miR-17-92 cluster miRs include: E2F1, E2F2, E2F3 (O'Donnell et al., 2005, Sylvestre et al., 2007), P21 (Inomata et al., 2009), anti-angiogenic thrombospondin-1 and connective tissue growth factor (Dews et al., 2006), proapoptotic Bim, and phosphatase and tensin homolog (PTEN) (Xiao et al., 2008). These genes are all involved in cell cycle regulation or apoptotic cell death, further supporting the importance of miR-17-92 cluster in T cell biology. Interestingly, while E2Fs (E2F1, E2F2, E2F3) are targets of miR-17-92, both E2F1 and E2F3 can directly bind the promoter of the *mir-17–92* cluster activating its transcription, establishing a negative feedback loop (Woods et al., 2007, Sylvestre et al., 2007). This negative feedback loop diminishes E2F activity thereby altering cell division/ cell death events.

Although retroviral expression of miR-17-92 cluster in the hematopoietic system accelerated the onset of cMyc-mediated lymphomagenesis (He et al., 2005), ectopic expression of miR-17-92 in T cells without cMyc over-expression does not induce cancer (Xiao et al., 2008). B and T cells in miR-17-92 transgenic mice exhibit enhanced proliferation and reduced cell death associated with down-regulation of Bim and PTEN (Figure 2, **left**). Furthermore, T cells transgenic for miR-17-92 demonstrate a lower threshold of activation following antigen recognition through TCRs (Xiao et al., 2008) and increased IFN-γ production compared with those from control mice (Xiao et al., 2008)(Kohanbash *et al.* submitted), suggesting that miR-17-92 may actually promote the type-1 skewing of T cells (Figure 2, **left**).

Further supporting the role of miR-17-92 in type 1 skewing is that it targets hypoxia-inducible factor (HIF)-1α in lung cancer cells (Taguchi et al., 2008), (Figure 2, **left**). In many cells HIF-1α expression provides an important adaptation mechanism for cells to accommodate to low oxygen tension (Sitkovsky and Lukashev, 2005, Semenza, 1998). However, unlike HIF-1α's role on survival of immune cells such as macrophages (Cramer et al., 2003), T cells do not depend on HIF-1α for survival, since activated T cells produce ATP primarily by glycolysis and, only when necessary, oxidative phosphorylation (Brand and Hermfisse, 1997). In T cells, HIF-1α appears to play predominantly an anti-inflammatory and tissue-protecting role by negatively regulating T cell function (Sitkovsky and Lukashev, 2005, Neumann et al., 2005, Eltzschig et al., 2004). T cell-targeted disruption of HIF-1α leads to increased IFN-γ secretion and improved effector functions (Kojima et al., 2002, Lukashev et al., 2006, Guo et al., 2009, Thiel et al., 2007). Thus, miR-17-92 expression in activated T cells may promote type-1 function of T cells in part through down-regulation of HIF-1α.

Transforming growth factor-β receptor (TGFBR)2 is a verified target of miR-17-92 in solid cancers (Volinia et al., 2006), and miR-17-92 expression in $CD34^+$ hematopoietic stem cells inversely correlates with the level of *TGFBR2* transcript (Merkerova et al.). It is therefore hypothesized that expression of miR-17-92 in T cells leads to down-regulation of TGFBR2, thereby rendering T cells more resistant to the immuno-suppressive effects of TGF-β, which is often expressed at high levels in a variety of cancers, including gliomas [reviewed by us in (Okada et al., 2009)] (Figure 2, **left**).

Our recent miR microarray analysis reveals that miR-17-92 is one of the most significantly over-expressed miR in murine type-1 T cells when compared with type-2 T cells (Kohanbash *et al.* submitted). Disruption of the IL-4 signaling through either IL-4 neutralizing antibody or knockout of STAT6 reverses the miR-17-92 cluster suppression in type-2 cells. Furthermore, T cells from tumor-bearing mice and glioma patients have decreased levels of miR-17-92 compared to cells from non-tumor bearing counterparts. Collectively, our data suggest that the type-2-skewing tumor microenvironment can induce the down-regulation of miR-17-92 expression in T cells, thereby diminishing the persistence of tumor-specific T cells and tumor destruction.

One major barrier for successful T cell–based cancer immunotherapy is the low persistence of tumor antigen (TA)-specific T cells in tumor-bearing hosts (Morgan et al., 2006, Pule et al., 2008). It seems promising to generate genetically modified TA-specific T cells *ex vivo* that are resistant to tumor-mediated immune suppression, promoting a robust and long-lived anti-tumor responses. miR-17-92 miRs could confer resistance to tumor-derived immunosuppressive factors and improve type-1 reactivity. Further characterization of the role of the miR-17-92 cluster in tumor antigen (TA)-specific CTLs is clearly warranted and may provide us with an ability to develop novel immunotherapy strategies with genetically engineered T cells. Additionally, identification of diminished miR-17-92 expression in the peripheral blood may emerge as an important biomarker in patients with malignancy.

miR-155

There is an interesting overlap when comparing the list of miRs that are implicated as oncomiRs and ones known to play important roles in immunity. Over-expression of miR-155 in hematopoietic cells induces malignancy (Costinean et al., 2006) or myeloproliferative disorder in mice and is associated with human acute myeloid leukemia (O'Connell et al., 2008). miR-155 also plays key roles in both innate and adaptive immune responses [reviewed in (Tili et al., 2008, Xiao and Rajewsky, 2009, Lindsay, 2008)]. It is critically important in the response to TLR ligands and PAMPs in our hands and that of others (O'Connell et al., 2009, O'Connell et al., 2007). miR155 directly regulates the SH2 domain containing inositol-5-phosphatase (SHIP)1 (O'Connell et al., 2009) (Figure 1). miR-155 seems to have important roles in differentiation of Th1 vs. Th2 T cells (Rodriguez et al., 2007, Thai et al., 2007). Disruption of miR-155 in naïve T cells results in polarized differentiation preferentially into Th2 cells, with substantial production of Th2 cytokines including IL-4, IL-5 and IL-10 (Rodriguez et al., 2007, Thai et al., 2007). In response to antigenic stimuli, miR-155-deficient T lymphocytes exhibit an impaired response and attenuated IL-2 and IFN-γ release (Rodriguez et al., 2007, Thai et al., 2007). It has been hypothesized that this bias results from miR-155 targeting of musculoaponeurotic fibrosarcoma (c-Maf), a transcription factor that is a potent transactivator of the IL-4 promoter (Rodriguez et al., 2007) (Figure 2, **left**). One could evaluate whether transgenic expression of miR-155 could reverse the cancer-induced skewing towards Th2 polarization and promote effective Th1 type anti-tumor immune responses. Indeed, a recent study has demonstrated that activation of T cells up-regulates miR-155 and over-expression of miR-155 in activated $CD4^+$ T cells promotes Th1 differentiation through the regulation of IFN-γRα chain (Banerjee et al., 2010), supporting this hypothesis. In addition, it would be interesting to determine whether it has a role in macrophage (M)1 and M2 differentiation pathways.

miR-181a

miR-181a augments the sensitivity of TCR-mediated T cell responses to peptide antigens Li et al., 2007). Regulation of T cell sensitivity by miR-181a enables mature T cells to abnormally recognize antagonists, the inhibitory peptide antigens, as agonists. Indeed, by measuring

changes in the intracellular Ca^{2+} transient and IL-2 release, miR-181a over-expression amplifies the strength and sensitivity of TCR-mediated activation (Li et al., 2007) (Figure 2, **left**). In addition, miR-181a, at least in part, regulates the positive and negative selection of T cells during thymic development. These effects are in part achieved by the down-regulation of multiple phosphatases, which leads to elevated steady-state levels of phosphorylated intermediates and a reduction of the TCR signaling threshold. The down-regulated phosphatases include the tyrosine phosphatases, Src homology 2 domain–containing protein-tyrosine phosphatase (SHP)-2, protein tyrosine phosphatase (PTP)-N22 and the ERK-specific, dual specificity phosphatases (DUSP)-5 and -6, which are all known to negatively regulate the TCR signaling pathway (Figure 2, **left**). Seemingly miR-181a acts as a rheostat by regulating protein phosphorylation levels. It would be interesting to determine whether ectopic over-expression of miR-181a and reduced phosphatase levels would lead to increased TCR signaling and a reduced T cell activation threshold in T cells recognizing nominally weak TAs.

miR-222 and -339

miRs expressed in tumor cells also regulate the recognition of tumor cells by immune cells, such as cytotoxic T lymphocytes (CTLs) (Figure 1, **right**). We recently reported that miR-222 and -339 in cancer cells down-regulate the expression of intercellular cell adhesion molecule (ICAM)-1, thereby decreasing the susceptibility of cancer cells to CTLs (Ueda et al., 2009). Dicer-deficient tumor cells up-regulate ICAM-1 expression, and exhibit enhanced susceptibility to antigen-specific lysis by CTLs, while expression of other immuno-regulatory proteins examined is not affected. A luciferase reporter assay demonstrate that miRs-222 and -339, both of which are down-regulated in Dicer-disrupted cells, directly interact with the 3’ untranslated region (UTR) of ICAM-1 mRNA. Modulation of Dicer or these miRs inversely correlates with ICAM-1 protein expression and susceptibility of U87 glioma cells to antigen-specific CTLs, whereas the ICAM-1 mRNA level remains stable. Immunohistochemical analyses and quantitative RT-PCR analyses of 30 primary glioblastoma tissues indicate that expression of Dicer or miR-222 is inversely associated with ICAM-1 expression. Our findings suggest that Dicer is responsible for the generation of miR-222 and miR-339, which suppress ICAM-1 expression on cancer cells, including glioma cells, thereby down-regulating the susceptibility of cancer cells to CTL-mediated lysis. The “antagomir”-mediated silencing of disease-associated miRs (Elmen et al., 2008) may be a novel and promising strategy for treatment of cancer waiting for clinical testing.

Conclusion

Tumors are equipped with multiple mechanisms to evade early events important in immunological surveillance, regulating their susceptibility to lysis. Based on our recent findings, these mechanisms appear to involve modulation of miRs that may have significant impacts on the function of anti-tumor T cells (i.e. down-regulation of miR-17-92 in tumor bearing hosts) (Kohanbash *et al.* submitted) and immune-mediated recognition of tumor cells (Ueda et al., 2009). On the other hand, recent advances in the field of miR research has providing us with valuable insights as to how we could improve the anti-tumor immune responses by engineering miRs. miR-17-92, -155 and -181a have extremely attractive biological properties that may be utilized for improvement of T cell-based cancer immunotherapy. Modulation of miRs in tumor cells may also represent viable and promising approaches. In each miR-based strategy, it is important to identify the target mRNAs, thereby gaining the firm understanding of the impact of miR-engineering therapeutic approaches. With this regard, we also have to keep it in mind that proliferating cells may express mRNAs with shortened 3' untranslated regions and fewer miR target sites (Sandberg et al., 2008). Lastly, modification of the miR transcript at any level of processing might positively or negatively impact its processing. This is the case in cancers where failure to process miRs at the Drosha

processing step leads to overall global levels of miRs (Thomson et al., 2006). Nevertheless, miR-targeting approaches will likely allow us to overcome some of our current challenges in cancer immunotherapy. Continued basic studies and translational efforts are warranted to further elucidate the roles of miR in both innate and adaptive immune responses.

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Abbreviations

miR	microRNA
DAMP	damage associated molecular pattern
PAMP	pathogen associated molecular pattern
DC1	type-1 dendritic cell
DC2	type-2 dendritic cell
Th1	type-1 T help cell
E2F	E2 transcription factor
PTEN	phosphatase and tensin homolog
IL	interleukin
TNF	tumor necrosis factor
TCR	T cell receptor
TGFBR	tumor growth factor beta receptor
TA	tumor antigen
CTL	cytotoxic T lymphocyte
SHIP	SH2 domain containing inositol-5-phosphatase
c-MAF	musculoaponeurotic fibrosarcoma
SHP	Src homology 2 domain–containing protein-tyrosine phosphatase
PTP	protein tyrosine phosphatase
DUSP	dual specificity phosphatases
ICAM	intracellular cell adhesion molecule
UTR	untranslated region
HMGB1	high-mobility group box
Pri-miR	primary microRNA
Pre-miR	precursor microRNA
IFN	interferon
Treg	regulatory T cell
STAT	signal transducer and activator of transcription
HIF	hypoxia inducible factor

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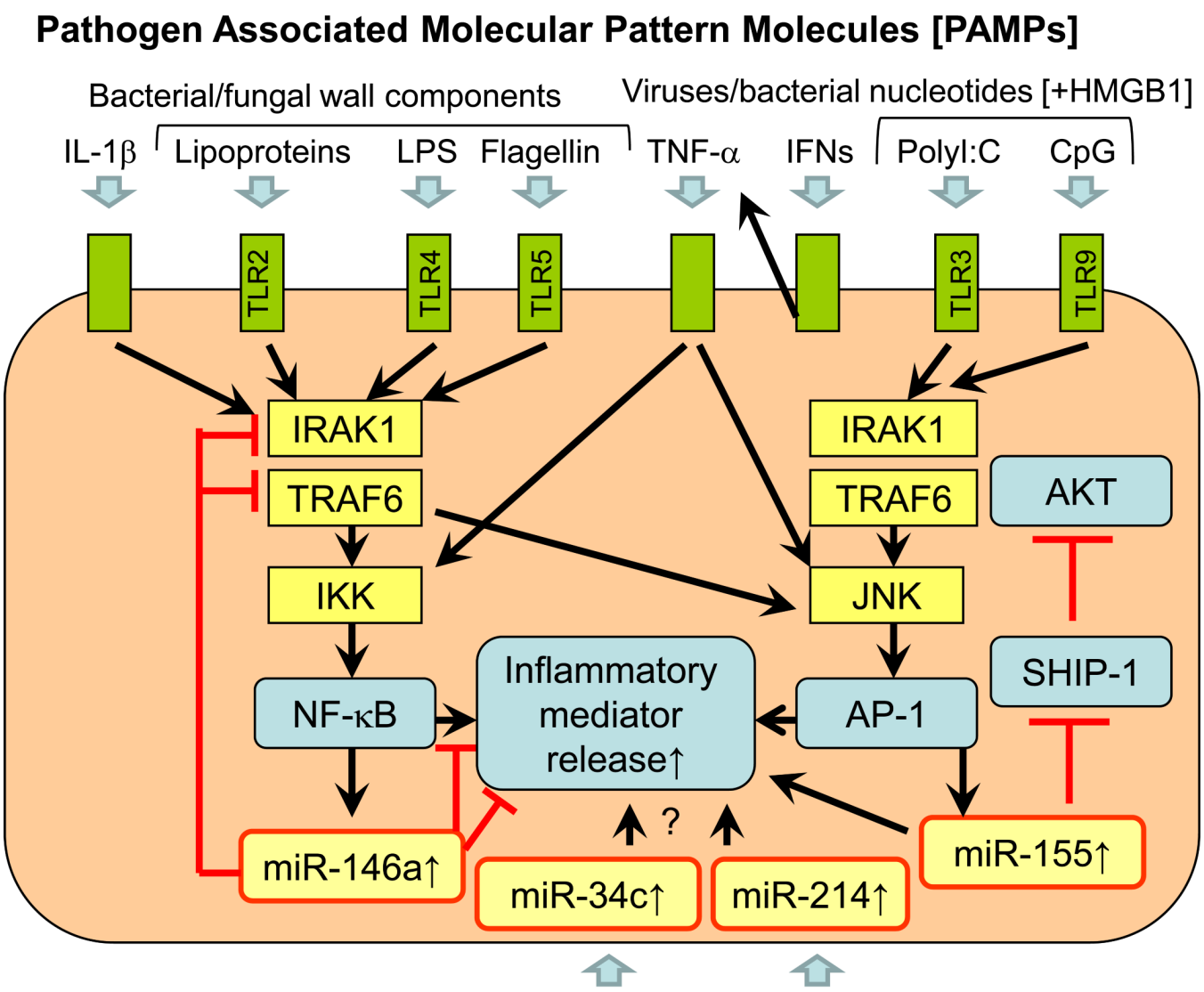

Figure 1. DAMPs and PAMPs mediate responses through induction of miRs

The illustration summaries the mechanisms by which individual miRs (red boxes) interact with target proteins (yellow boxes) to regulate cell responses (blue circles) in macrophages. Stimulation (↑) or inhibition (T) should be determined by following how the changes in the individual miR expression (red squares) impacts on the indicated biological response (blue circles). In our studies almost all of the DAMPs up-regulate expression and miR155 in macrophages (O'Connell et al., 2007, O'Connell et al., 2009). Baltimore's group has shown that miR 146 serves as a block on many macrophage functions (Taganov et al., 2006). Recently we have demonstrated that miR 34c is up-regulated by High-mobility group box (HMGB)1 expressing cells, releasing DAMPs and miR 214 independently of expression of HMGB1 (Unlu S et al, submitted). AP-1, activator protein; IRAK, IL-1 receptor activated kinase; JNK, c-jun N-terminal kinase; NF, nuclear factor; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor. From Lindsay, M.A. (2008) MicroRNAs and the immune response. Trends in Immunology *29*, 343-351 with modifications.

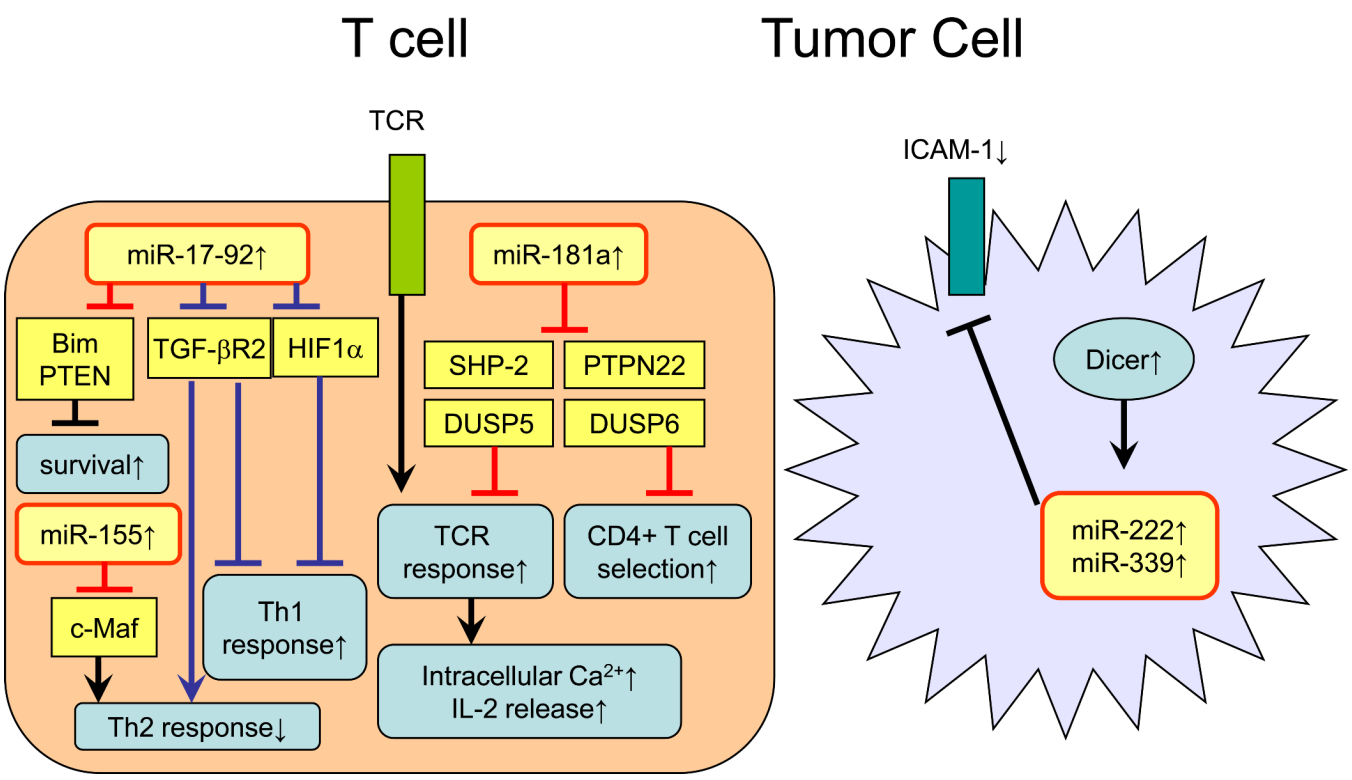

Figure 2. Role of microRNAs (miRs) in the regulation of the immune response

The illustration summaries the mechanisms by which individual miRs (red boxes) interact with target proteins (yellow boxes) to regulate cell responses (blue circles) in T cells (left) and tumor cells (right). Stimulation (↑) or inhibition (T) should be determined by following how the changes in the individual miR expression (red squares) impacts on the indicated biological response (blue circles). Black arrows and red lines are effects that have been demonstrated to operate in the indicated cell type. Blue arrows and lines are ones that have been shown to operate in tumor cells but yet to be proven in T cells. DUSP, dual specificity phosphatase; HIF, hypoxia-inducible factor; Maf, musculoaponeurotic fibrosarcoma; PTEN, phosphatase and tensin homolog; SHP, PTP, protein tyrosine phosphatase; Src homology 2 domain–containing protein-tyrosine phosphatase; TCR, T cell receptor; TGF, transforming growth factor. From Lindsay, M.A. (2008) “MicroRNAs and the immune response”. Trends in Immunology *29*, 343-351 with modifications.