



Mini-review

MicroRNAs in type 2 immunity

Kristina Johansson, Julie Weidner, Madeleine Rådinger^{*}

Krefting Research Centre, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden



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ABSTRACT

Type 2 immunity drives the pathology of allergic diseases and is necessary for expulsion of parasitic worms as well as having important implications in tumor progression. Over the last decade, a new research field has emerged describing a significant link between type 2 immunity and cancer development, called AllergoOncology. Thus, type 2 immune responses must be carefully regulated to mediate effective protection against damaging environmental factors, yet avoid excessive activation and immunopathology. Regulation of gene expression by microRNAs is required for normal behavior of most mammalian cells and has been studied extensively in the context of cancer. Although microRNA regulation of the immune system in cancer is well established and includes type 2 immune reactions in the tumor microenvironment, the involvement of microRNAs in these responses initiated by allergens, parasites or other environmental factors is just emerging. In this review, we focus on recent advances which increase the understanding of microRNA-mediated regulation of key mechanisms of type 2 immunity.

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1. Introduction

Type 2 immune responses are generally referred to as mechanisms driven by interleukin-4 (IL-4), IL-5 and IL-13 [1]. They are produced by T helper type 2 (T_H2) lymphocytes and type 2 innate lymphoid cells (ILC2) in response to a variety of environmental factors including allergens, helminths, virus, bacteria, fungi and chemical irritants (Fig. 1) [2]. Type 2 responses involve multiple immune cells of both the innate and adaptive immune system as well as non-hematopoietic epithelial, stromal and smooth muscle cells. MicroRNAs (miRNAs) are required for normal function of both hematopoietic and non-hematopoietic cells and are involved in fundamental biological processes such as development, proliferation, apoptosis, tumorigenesis and immune reactions [3–7]. They consist of short non-coding RNAs that mediate sequence-specific repression of target messenger RNAs (mRNAs) inhibiting gene expression at the post-transcriptional level [8]. The first miRNA to be identified was *lin-4* in the round worm *C. elegans* in 1993 [9]. Since then, the field has exploded with more than 1800 validated human miRNAs and over 28,000 total miRNAs identified in metazoans [10].

A single miRNA can have multiple mRNA targets depending on cell type, tissue or even cell status. Alternatively, several different miRNAs can regulate a single mRNA, expanding and complicating the role of miRNAs in the cell. But this complexity also represents the strength of miRNAs which often regulate functionally related gene transcripts involved in common biological pathways. Indeed, altered expression of a single miRNA can change the course of an inflammatory process and affect disease progression [11–13]. This, of course, makes miRNAs highly attractive for therapeutic intervention, where the miRNAs themselves, or downstream gene products, might be targeted. For example, specific miRNAs that regulate type 2-mediated immunosuppression in the tumor microenvironment might be altered to induce tumor surveillance, and identification of miRNAs involved in allergic sensitization and maintenance of chronic inflammation might be utilized in novel prevention or treatment strategies of allergic diseases in the future. In recent years, a new field of study, referred to as AllergoOncology has emerged [14–16]. AllergoOncology recognizes that there are significant links between cancer and type 2 responses to allergens, pathogens or parasites. Furthermore, a number of major players in type 2 immunity, such as immunoglobulin E (IgE)-responsive cells, T regulatory cells, and macrophages have been reported to infiltrate the tumor microenvironment. With the knowledge gained regarding immune responses in allergy and asthma, correlations can hopefully be made to the role of these immune cells in cancer.

^{*} Corresponding author. University of Gothenburg, Krefting Research Centre, Box 424, SE-405 30, Gothenburg, Sweden.

E-mail address: madeleine.radinger@gu.se (M. Rådinger).

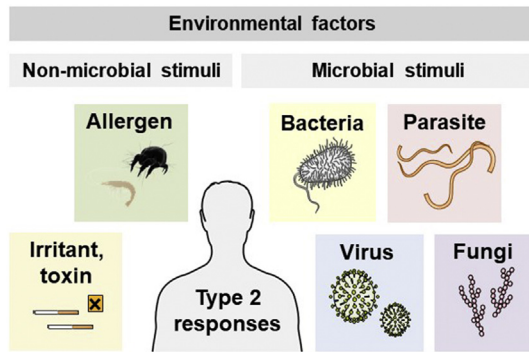


Fig. 1. Type 2 immune responses are triggered by a variety of microbial or non-microbial environmental factors such as parasites, viruses, bacteria, fungi, allergens and chemical irritants.

Perhaps future studies may even delve into differences in miRNA signatures between immune cells within the tumor microenvironment and the rest of the body. In this review, we discuss key players of innate and adaptive type 2 immune responses and focus on specific miRNAs that are critical in the regulation of these events (summarized in Fig. 2).

1.1. miRNA biosynthesis

The miRNA biosynthesis pathway has been extensively studied and described in great detail [17–19]. Briefly, miRNAs are generally transcribed via RNA polymerase II into stem loops with single stranded miRNA ends known as primary miRNA (pri-miRNA). Pri-miRNAs are processed into precursor miRNAs (pre-miRNA) in the nucleus by the enzymes Drosha and DiGeorge syndrome critical

region 8 (DGCR8) before their transport to the cytoplasm via the exportin-5 complex [20]. Once in the cytoplasm, the pre-miRNAs have their single stranded ends trimmed before being bound by the enzyme Dicer, which cleaves pre-miRNA into -3p and -5p pieces. The single, approximately 18–24 nucleotide strand (either -3p or -5p) is then loaded into the RNA-induced silencing complex (RISC) [8]. It was believed that the unloaded strand was originally degraded, but recent studies have shown that, in some cases, both -3p and -5p miRNAs bind to various cellular targets [21]. The approximately 8 nucleotide, highly conserved seed sequence of the miRNA then binds to a target mRNA, ultimately leading to translational inhibition or decay. Originally thought to bind solely to the 3' end of the mRNA transcript, miRNAs have now been shown to bind within the 5' end and/or coding regions of the target [22,23]. Furthermore, in some cases miRNAs can also promote translation of targets by binding to elements in the 5' untranslated region (UTR) of the mRNA, thus adding to the complexity of miRNA-mediated regulation [24,25].

2. miRNA regulation of innate type 2 responses

To maintain a balance between immune homeostasis and immune activation is particularly challenging at mucosal barriers due to the constant exposure to potentially harmful stimuli. Over the past decade, interest has been peaked regarding the epithelium and the discovery of a subset of cells, which respond to epithelial-derived factors, known as ILC2s. Epithelial stress caused by for example virus, allergens or chemical irritants triggers the release of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) which activates rapid and robust production of type 2 cytokines by ILC2s [26,27].

2.1. Epithelium

The epithelium is increasingly recognized as a site for initiation

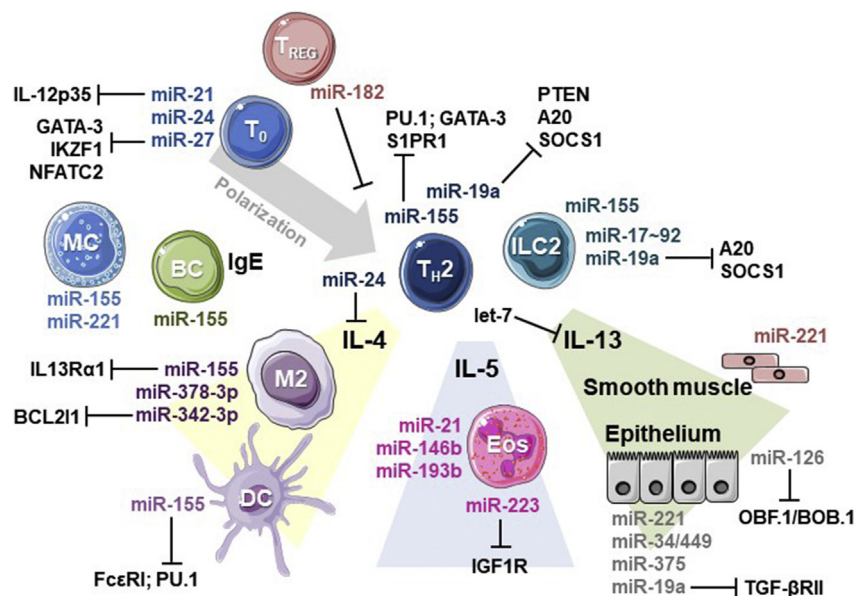


Fig. 2. Several miRNAs have been identified that regulate type 2 immune responses. The miR-17–92 cluster regulates ILC2 homeostasis, where specifically miR-19a has been found to target A20 and SOCS1. miR-221 regulates proliferation in smooth muscle cells and epithelial to mesenchymal transition. miR-221 also regulates immunoglobulin E (IgE)-mediated mast cell (MC) degranulation and cell cycle in resting cells. miR-34/449 regulates epithelial cell differentiation, and miR-126 and miR-19a are expressed in bronchial epithelium, while miR-375 regulates epithelial properties in the gut. miR-155 was originally identified in B cells (BC), but is known to regulate functions of various immune cells including ILC2s, T_H2 cells (SP1PR1, GATA-3 via PU.1), macrophages (IL13Rα1), dendritic cells (DC; FcεRI receptor via PU.1) and mast cells. miR-378-3p represses IL-4-driven macrophage polarization, and miR-342-3p regulates apoptosis in macrophages by targeting BCL2L1. miR-223 limits eosinophil proliferation by targeting IGF1R, whereas miR-21, miR-146b and miR-193b promote eosinophil differentiation. miR-21 regulates T_H2 cell polarization from naïve T cells (T₀) by targeting IL-12p35. miR-19a regulates T_H2 cell responses by targeting of PTEN, A20 and SOCS1, and miR-182 regulates T regulatory (T_{REG})-mediated suppression of T_H2 responses. Let-7 directly targets IL-13, and miR-24 directly targets IL-4. miR-24 and miR-27 inhibit T_H2 differentiation where miR-27 targets GATA-3, IKZF1 and NFATC2.

of type 2 immune responses [28]. The importance of miRNA-mediated regulation of epithelial cells was demonstrated in early studies using targeted deletion of the miRNA-processing enzyme Dicer [29]. Dicer knockout cells are incapable of converting precursor miRNAs into mature, functional miRNAs, resulting in global miRNA deficiency. As a consequence, severe morphological and functional defects have been reported in miRNA deficient epithelium of the skin [30] intestine [31] and airways [29].

An important, multifunctional cytokine with several roles at mucosal surfaces is transforming growth factor β (TGF- β) [32]. For instance, it regulates epithelial and smooth muscle cell proliferation which are hallmarks of tissue remodeling in asthma [33]. Indeed, airway smooth muscle cells from severe asthmatics respond with increased proliferation and higher IL-6 secretion in response to TGF- β stimulation compared to cells obtained from mild asthmatics and healthy controls [34]. In addition, miR-221 was significantly increased in severe asthmatics and inhibition of miR-221 resulted in reduced proliferation and IL-6 release. miR-221 has also been found to facilitate TGF- β -induced epithelial to mesenchymal transition, a process that could be reversed by blocking miR-221 activity [35].

Detailed studies in mice revealed that lack of pulmonary epithelial cell-derived TGF- β results in reduced airway inflammation upon inhaled allergen challenge [36]. This was explained by significantly reduced frequency of IL-13⁺ ILC2s, but not IL-13⁺ T_H2 cells, in allergen challenged mice with a TGF- β deletion restricted to airway epithelial cells [36]. Furthermore, in the aforementioned study, Denney et al. demonstrated that TGF- β induced migration in ILCs expressing TGF- β receptor 2 (TGF- β RII). Interestingly, TGF- β RII has been reported to be a direct target of miR-19a in human bronchial epithelium. The expression levels of miR-19a were higher in epithelial cells from severe asthmatic subjects compared to mild asthmatics and healthy controls [37]. In mild asthma, the epithelium is damaged and fails to proliferate and repair whereas severe asthma is characterized by excessive proliferation leading to thickening of the airway wall. There are likely different regulatory mechanisms at play since disruption of TGF- β signaling has been shown to both promote and suppress epithelial proliferation [38,39]. IL-13 production by ILC2s may also have implications in regulation of cancer progression where IL-33 administration has been found to promote tumor growth and metastasis in a murine breast cancer model [40]. Tumor progression was reported to correlate with increased numbers of intra-tumoral IL-13⁺ ILCs and IL13R⁺ myeloid-derived suppressor cells that produced TGF- β . However, our understanding of the involvement of ILC subsets in cancer progression is currently limited and requires further investigation.

In asthma, ILC2-derived IL-13 was recently shown to promote bronchial epithelial barrier leakiness, an important pathological feature of the disease [41]. The authors demonstrated the importance of IL-13 as the barrier function could be restored by neutralization of IL-13. Interestingly, a study of airway epithelial miRNAs identified decreased expression in members of the miR-34/449 family in asthmatic compared to healthy subjects [42], and miR-34/449 was found to regulate epithelial cell differentiation *in vitro*. Furthermore, IL-13 stimulation had a strong and consistent effect on the miRNA expression in normal bronchial epithelial cells that induced mucus metaplasia and recapitulated the repression of miR-34/449 family members that were observed in asthma [42]. In addition to the immune system of the airways, the effects of IL-13 on miRNA expression have been studied in intestinal immunity where miR-375 expression together with TSLP increased upon IL-13 signaling in intestinal epithelial cells [43]. The phenotype of miR-375 deficient mice mimicked morphological and immunological defects of mice lacking functional Dicer in the intestinal

epithelium as mentioned previously. These Dicer knockout mice were unable to mount an effective type 2 response upon helminth infection and the study identified miR-375 as a key regulator of epithelial properties that are necessary to amplify type 2 responses in the gut.

2.2. miRNA regulation of ILC2s

Although ILC2s have received much attention in recent years, miRNA-mediated regulation of these cells is largely unexplored and, so far, only two studies have reported a role of miRNAs in ILC2s [44,45]. We have recently found that miR-155 is important for ILC2-mediated inflammation in mice. Lung ILC2s increased miR-155 expression upon IL-33-mediated activation *in vitro* and miR-155 deficient ILC2s demonstrated decreased IL-13 production and lower proliferative capacity in response to IL-33 administration *in vivo* [44]. However, direct targets of miR-155 have not yet been identified in ILC2s. For instance, wild type or miR-155 deficient ILC2s did not express the previously established miR-155-target PU.1 which is an inhibitor of GATA-3; the master regulator of ILC2s [44]. However, other direct targets of miR-155 such as suppressor of cytokine signaling 1 (SOCS1) [46] was identified as a regulator of ILC2 cytokine production by Singh et al. as mentioned below [45], and might be a target of miR-155 in ILC2s. Although, the exact molecular mechanisms remain unclear, further studies are warranted to determine the role of miR-155 and other miRNAs in ILC2 biology. This includes investigation into the involvement of miRNAs in the rapid and proportionally large production of pro-inflammatory mediators by ILC2s compared to other lymphocytes (e.g. CD4⁺ T cells [47]) as well as the role of miRNAs in ILC2 development and maintenance of lineage stability or directed plasticity under homeostatic versus inflammatory conditions.

A recent study compared miRNA expression profiles at various activation states of ILC2s and T_H2 cells isolated from murine lungs [45]. On a global scale, the analysis revealed a significant overlap of miRNAs expressed in all cells examined. Increased expression of miR-21a, miR-98 and miR-155 and decreased expression of let-7c, miR-151 and miR-203 were found in both ILC2s and T_H2 cells following *in vitro* activation. However, a few miRNAs were differentially expressed in ILC2s compared to T cells, this list included miR-126a, miR-134, miR-409 and miR-541. Importantly, by generating an *in vivo* model with a selective ILC2-deficiency for the cellular component DGCR8, which is necessary for miRNA biogenesis, the authors demonstrated that miRNAs are essential for ILC2 homeostasis [45]. Specifically, the miR-17–92 cluster had important functional implications in ILC2s, as cells lacking this family of miRNAs displayed reduced cytokine production and growth in allergen-induced lung inflammation [45]. The miR-17-92 cluster family member miR-19a was found to negatively regulate tumor necrosis factor alpha-induced protein 3 (TNFAIP3) which encodes A20, and SOCS1 in the ILC2s. These genes have previously been reported to inhibit IL-5 and IL-13 cytokine production [12,48,49].

2.3. M2 macrophages

Macrophages activated by type 2 cytokines are characteristic of anti-helminthic immune responses and tissue repair. These M2, or alternatively activated macrophages antagonize the function of classical pro-inflammatory M1 macrophages which are induced by interferon gamma (IFN- γ) and bacterial lipopolysaccharide (LPS) [50]. In contrast, IL-4 activates the IL4R α /Janus kinase (JAK)/signal transducer and activator of transcription (STAT6) and phosphoinositide 3-kinase (PI3K) pathways which drive alternative macrophage polarization [51]. A number of miRNAs have been identified

to regulate genes involved in alternative macrophage activation (reviewed in [52]). For instance, extensive studies of miR-155 have revealed multiple targets in macrophages including IL13R α 1 which inhibits STAT6-dependent M2 polarization [53]. In addition, miR-155 has also been found to favor the M1 phenotype by inhibiting negative regulators of M1 polarization [54–56]. Furthermore, miR-125b-5p, miR-199b and miR-378-3p were expressed at elevated levels in alternatively activated macrophages in an *in vivo* mouse model of nematode infection [57]. That study, specifically showed miR-378-3p repressed IL-4-driven macrophage proliferation via modulation of the PI3K/AKT signaling pathway [57]. A recent study of IL-4-responsive miRNAs identified induction of miR-193b and miR-342-3p and repression of miR-99b and miR-125a-5p in human macrophages [58]. The same expression pattern was found *in vivo* in mice upon parasite infection. Interestingly, miR-342-3p was shown to decrease the viability of macrophages by inducing apoptosis. The anti-apoptotic target BCL2 Like 1 (BCL2L1) was identified and it was suggested that increased miR-342-3p partially controls excessive macrophage proliferation in type 2 responses [58]. Allergic inflammation and tumor development are also characterized by a predominance of alternative macrophage activation. In both conditions, miR-124, miR-324-5p and miR-511-3p have been shown to regulate macrophage functions [59–62].

2.4. IgE-responsive cells: mast cells, dendritic cells and eosinophils

Immune reactions mediated by immunoglobulins involve a complex interplay of innate and adaptive immune cells, including antigen presenting cells such as dendritic cells and T and B lymphocytes. The IgE isotype-switching in B cells develops under the influence of IL-4 and elevated circulating IgE antibodies are a hallmark of allergy [63]. IgE acts on a range of potent effector cells of the innate immune system bearing the high affinity IgE receptor Fc ϵ RI such as mast cells, eosinophils and basophils [64]. miR-155 has been found to regulate Fc ϵ RI-mediated functions of both mast cells and dendritic cells. In dendritic cells, the expression of the Fc ϵ RI receptor itself was recently found to be downregulated by miR-155 in response to toll-like receptor signaling via the transcription factor PU.1 [65]. Mast cells lacking miR-155 expression have demonstrated enhanced degranulation and cytokine release (TNF- α , IL-6 and IL-13) upon IgE-mediated stimulation [66]. miR-221 has also been reported to promote mast cell degranulation [67]. However, miR-221 seems to have additional roles in mast cells, contributing to the regulation of cell cycle and actin cytoskeleton in resting cells [67].

IL-5 is an important signaling factor for eosinophil development and survival. Negative regulation of IL-5 is poorly understood and currently only a single study has reported post-transcriptional regulation of this cytokine. Increased expression of IL-5 was mediated through direct interaction of miR-1248 with the IL-5 transcript [68]. Studies linking miRNA regulation to eosinophils have found that miR-223 is expressed under eosinophil differentiation and that targeted ablation of miR-223 resulted in increased proliferation of eosinophil progenitors [69]. Inhibition of the miR-223 target gene, insulin-like growth factor 1 receptor (IGF1R), which is known to stimulate cell proliferation and inhibit programmed cell death, reversed the effect in miR-223 deficient cells. In contrast, miRNAs that have been found to be positively associated with eosinophil differentiation include miR-21, miR-146b and miR-193b [70–72].

3. miRNA regulation of adaptive type 2 responses

T_H2 lymphocytes are crucial for orchestration of adaptive type 2 immune responses elicited by infectious parasites and allergens.

Indeed, allergic asthma has long been considered a T_H2-mediated disease, in which the signature effector cytokines IL-4, IL-5 and IL-13 drive persistent eosinophilic airway inflammation, mucus overproduction, airway hyper-responsiveness and remodeling of the airway wall [73]. However, reports in recent years have highlighted the complex interplay of innate and adaptive immune cells that participate in both the initiation and maintenance of adaptive type 2 responses. For instance, studies of ILC2s in models of airway inflammation induced by papain, a protease allergen, have found that ILC2s promote adaptive T cell responses by producing IL-13 that activates dendritic cells to migrate into lung draining lymph nodes where they polarize naïve CD4⁺ T cells into the T_H2 phenotype [74]. A study using a helminth infection model reported impaired T_H2 responses in the absence of ILC2s [75], which further supports the idea that ILC2s promote adaptive type 2 responses. However, the opposite relationship between ILC2s and T cells have also been reported where the induction of ILC2s was critically dependent on the activation of CD4⁺ T cells in a model of house dust mite (HDM) allergen-induced airway inflammation [76].

3.1. T cells

Early profiling studies in murine models have identified cell-specific miRNA expression patterns, which suggest a role for miRNAs in lineage commitment and T cell effector functions [77,78]. Thus, not surprisingly, deletion of essential components of the miRNA biogenesis pathway have revealed a critical role for miRNAs in T cell activation and function [79]. Interestingly, increased differentiation towards effector T_H cell phenotypes and cytokine production was seen in naïve CD4⁺ T cells lacking miRNAs, suggesting an important role for miRNAs in naïve T cell maintenance. However, how this process is regulated remains largely unknown and more in-depth mechanistic work will be needed.

3.2. T_H2 cells

The miRNAs that take part in T_H2 cell differentiation are poorly understood as compared with T_H1 cell differentiation, which has been more thoroughly studied. However, there are specific miRNAs that have been found to participate in polarization of adaptive type 2 immune responses. For example, miR-21 has been demonstrated to be upregulated in several models of experimental allergic asthma, and is believed to regulate the T_H1 versus T_H2 balance through targeting of IL-12p35 expression [80]. Furthermore, allergen challenged miR-21 deficient mice had reduced airway eosinophilia and significantly decreased levels of the T_H2 cytokine IL-4 in bronchoalveolar lavage fluid (BALF), whereas the T_H1 cytokine IFN- γ was increased. On the contrary, overexpression of miR-21 promoted *in vivo* differentiation of T_H2 cells [81] and miR-21 has been identified as the most upregulated miRNA in human patients with eosinophilic esophagitis, a disease characterized by IL-13 overproduction and a strong T_H2 inflammation of the esophagus [82]. Interestingly, miR-21 levels inversely correlate with IL-12p35 levels in these patients and, thus, highlighted miR-21 as a key regulator of IL-12 and T cell polarization. In addition, miR-21 has also been shown to be increased in asthmatic children and it has been suggested to be used for differentiating steroid responsiveness in these patients [83].

In murine models of HDM-induced asthma, inhibition of miR-126 or miR-145 resulted in a reduced T_H2 response as well as a decrease in eosinophil recruitment to the airways and mucus hypersecretion [11,84]. However, miR-126 was highly expressed in the airway wall and, to a much lesser extent, in T cells, suggesting that miR-126 had an indirect effect on T_H2 cells in this model via the transcription factor Oct binding factor 1 (OBF1) or B cell Oct

binding protein 1 (BOB.1) [84]. OBF.1/BOB.1 is a critical regulator of antibody production, but also a negative regulator of PU.1 which has been reported to inhibit the T_H2 transcription factor GATA-3. Interestingly, type 2 cytokines promote tumor metastasis and contribute to chemoresistance, and miR-126 has been shown to promote tumor angiogenesis via a T_H2 -dependent IL-13 release mechanism, in a model of breast tumor metastasis [85]. Furthermore, high miR-126 expression in acute myeloid leukemia patients has been associated with higher incidence of relapse and, additionally, poor survival [86].

3.3. B cells

miR-155 is located in the B cell integration cluster (BIC) gene and was originally described in Burkitt's lymphoma and in human B cell lymphoma patients where overexpression of miR-155 resulted in B cell malignancy [87,88]. Several miRNAs support differentiation of B cells to plasma cells, a cell type of critical importance in type 2 immune responses by its ability to produce IgE antibodies that act on FcεRI-expressing cells as previously discussed. Both miR-155 and miR-182 are required for antigen-specific IgG1 antibody production by B cells, and miR-155-deficient B cells demonstrate reduced germinal center responses after immunization. Furthermore, expression of the transcription factor PU.1 in B cells has been shown to be elevated in the absence of miR-155, where miR-155 was suggested to regulate T cell-dependent antibody responses in a B cell intrinsic manner [89–91]. Additionally, miR-182 has been found to be important for effector T cell responses and is required in T regulatory cells in order to suppress T_H2 -mediated inflammation [92,93].

4. miRNA regulation of allergy and asthma *in vivo*

We and others have demonstrated that miR-155 promotes pathogenic type 2 immune responses *in vivo* in mouse models of asthma [44,94,95]. miR-155 was significantly upregulated in lung tissue in a mouse model of ovalbumin (OVA)-induced allergic airway inflammation. Indeed, allergen-challenged miR-155 deficient mice displayed diminished T_H2 responses, resulting in reduced airway eosinophilia. These mice also demonstrated increased expression of the transcription factor PU.1 locally in the airways, opening the possibility that the absence of miR-155 inhibits GATA-3 in this model [94]. Furthermore, in a mouse model of HDM-induced asthma, miR-155 was shown to promote airway hyper-responsiveness and cell recruitment in part through the regulation of the direct target Sphingosine-1-phosphate receptor 1 (S1PR1) [95]. In human asthma, miR-155 was shown to be down-regulated in exhaled breath condensates compared to healthy control samples [96]. We have similarly found that miR-155 was significantly lower in induced sputum samples from subjects with allergic asthma compared to healthy controls [97]. Furthermore, sputum samples isolated from allergic asthmatics in and out of pollen season revealed that the level of miR-155 in sputum lymphocytes was downregulated during pollen season [97]. However, further studies in human asthmatics are required to delineate the mechanisms behind the reduction of miR-155 expression in human asthma.

One of the first studies describing functionality of a miRNA in human asthma pathogenesis was by Simpson et al. demonstrating a role for miR-19a in promoting type 2 cytokine production in airway T_H cells. Notably, miR-19a was significantly higher in BALF T_H2 cells from asthmatic subjects compared to healthy controls, and was shown to promote IL-13 production by T_H cells by direct targeting of the inositol phosphatase PTEN, the signaling inhibitor SOCS1 and A20 [12]. Previous studies have shown that the miR-17–92 cluster

promotes T cell survival and proliferation [98], however Simpson et al. suggest that upregulation of miR-19a in human asthma, may be an indicator and a cause of increased T_H2 cytokine production in the airways. In addition to miR19a, the let-7 family of miRNAs, which are ubiquitously expressed in many cells, was shown to regulate the expression of IL-13 through directly targeting the 3'UTR of the IL-13 transcript *in vitro* [99]. In a mouse model of asthma, intranasal administration of a let-7 miRNA mimic led to reduced IL-13 expression and resolution of allergic airway inflammation [100]. Although let-7 family members target IL-13, contradictory results from *in vivo* models have been reported where administration of let-7 inhibitors decreased airway inflammation [99]. In human asthma, expression of let-7 family members was decreased in both BALF and in exhaled breath condensates from asthmatics compared to healthy controls [96,101], which might suggest an inhibitory role of let-7 in human asthma immunopathogenesis.

Several miRNAs have been demonstrated to inhibit T_H2 activity *in vivo* [95,102,103]. The miR-23–27–24 cluster were recently shown to regulate T_H2 differentiation and effector function in mice. Two independent reports identified both miR-24 and miR-27 to inhibit T_H2 differentiation and IL-4 production [102,103]. miR-27 was shown to repress GATA-3, IKAROS Family Zinc Finger 1 (IKZF1) and Nuclear Factor of Activated T-cells 2 (NFATC2), all of which are upstream mediators of IL-4, while miR-24 directly targeted the 3'UTR of IL-4. Notably, as miR-24 and miR-27 were found to limit IL-4 production in T cells, the deletion of the miR-24–27 cluster promoted T_H2 dependent responses *in vivo* [103]. In a similar fashion, deletion of miR-146a has been found to result in increased type 2 immune response upon airway exposure to HDM in an *in vivo* mouse model of asthma [95]. Interestingly, polymorphisms in miR-146a were found to be associated with human asthma and elevated levels of miR-146a have been observed in T cells from patients with T cell-driven leukemia [104,105]. In summary, an increasing body of evidence support that individual miRNAs have distinct and critical effect on type 2 immune responses of relevance in a variety of immunological contexts.

5. Extracellular miRNAs

Since the discovery of stable miRNAs in exosomes from blood [106], there has been an increased interest in using miRNAs as biomarkers and in potential future therapies. Circulating miRNAs are characterized due to their resistance to a number of elements, such as extreme temperature, pH and RNases, though this is perhaps unsurprising as miRNAs are bound to proteins or within exosomes [106–108]. Furthermore, transport of miRNAs in exosomes point to an important mode of cell-cell communication, potentially over long distances in the case of circulating miRNAs [109–111]. To date, extracellular miRNAs have been observed in several bodily fluids including, but not limited to, blood, plasma, serum, sputum supernatants, bronchial lavage, and urine. One of the biggest advantages to studying miRNAs in these fluids is the ease and availability of access in human subjects. Additionally, circulating miRNAs can be stable for years with proper storage, meaning that samples collected from previous studies could be utilized in the identification and discovery of new potential biomarkers [112–116]. With the advent of circulating miRNAs, researchers performing translational studies could begin to relate mouse to man.

Most recently, several groups have taken advantage of examining circulating miRNAs in respect to a host of different diseases. Circulating miRNAs have been examined in numerous cancers, type II diabetes, neurodegenerative diseases, lung diseases, such as asthma, and cardiovascular diseases [42,117–120]. It is

apparent that since the first miRNA–cancer association was found in 2002 [121], there has been an explosion of interest in the field with almost half of all published papers on miRNAs being associated with cancer [122]. Circulating miRNAs tend to be very tissue specific and, thus, can act as biomarkers for different cancers, potentially originating from the spot of metastasis [106,107,123–128]. The well-studied miRNA, miR-221, has been identified in several studies examining circulating miRNAs in cancer. In pancreatic cancer, it was shown to be consistently overexpressed in tissue and upregulated in plasma of those patients with malignant, but not benign tumors [129]. Additionally, colorectal cancer patients with high levels of circulating miR-221 were shown to have high chance of survival [130]. A cluster of miRNAs, including miR-379 and miR-409-3p, was shown in mice, and later confirmed in humans, to be upregulated in the serum of patients with prostate cancer, breast cancer and lung adenocarcinoma [131–133]. In ovarian cancer, it was found that many of the circulating miRNAs, including miR-21, -141, -200a, -200b, -200c, -203, -205, and -214, upregulated in the disease were found in exosomes extracted from blood serum and plasma [126]. Exosomes are released from a variety of cell types including tumor and immune cells and the composition of exosomes changes based on the cell of origin [134]. Several studies have suggested a cross-talk between immune cells and cancer via miRNAs. For instance, miR-223 has been reported to be transferred via exosomes from activated M2 macrophages into breast cancer cells and promotes cell migration [135] and tumor-derived miR-214 has been found to be capable of suppressing the host's immunity [136]. Studies of miRNA and cancer continue to be a hot topic in the field and miRNA signatures for specific diseases are an important step in their development as reliable clinical biomarkers.

Recent investigations into asthma have utilized the current tools available in miRNA technology and subsequent analysis to produce quite elegant and informative studies. The first study to establish that circulating miRNAs could be used to distinguish different asthma groups using *in vivo* and *in silico* techniques came from Panganiban and colleagues. Five different groups consisting of a total of 30 differentially expressed and detectable miRNAs were identified in plasma of asthmatics and allergic rhinitis patients. From these subjects, a signature of 20 miRNAs were found to increase in patients with high eosinophil levels including the previously discussed miR-223, miR-126, miR-21 and let-7b [137]. Milger and colleagues performed a translational study, looking at miRNA from the plasma of two different murine models, the OVA and HDM models of asthma, in order to take advantage of sensitization stages and aeroallergen exposure. After identifying candidates via microarray and further validation by qPCR, 13 murine miRNAs were chosen for further study in well-defined human asthmatic subjects. The group found miRNAs previously identified as implicated in asthma pathogenesis (miR-223) or as a biomarker (miR-21), as well as potentially new miRNAs validated by qPCR and ready for further study [138]. Kho et al. recently performed a thorough study to identify biomarkers from circulating miRNAs found in serum from patients with childhood asthma [139]. Through microarray studies and comparison of their results to previously performed studies, the group was able to identify circulating miRNAs that were associated with different lung function parameters. Panganiban et al. took their study a step further to not just examine differential miRNA expression in serum, but also to identify IL-5 as a target mRNA for their top miRNA hit [68]. As previously mentioned, miR-223 has been a critical miRNA in beginning to understand cell-to-cell communication [140]. During pulmonary inflammation, neutrophils were shown experimentally to transfer miR-223 to epithelial cells, leading to a downregulation of PARP-1, a pro-

inflammatory gene, which in turn protected the cell against both inflammation and injury. These are just a few studies to describe the recent work that has been performed in examining the role of extracellular miRNAs in disease. Certainly there is much more work to be done in the field of biomarkers, circulating miRNAs and their role in disease.

6. Future perspectives

As changes to miRNA expression are seen in numerous diseases from cancers to respiratory diseases, it is becoming more apparent that they might be used as viable biomarkers for new therapeutic treatments [141]. This work has already started with the advent of antagomirs, siRNAs and miRNA mimics which may be useful potential future treatments to restore homeostasis [142]. Antagomirs are engineered oligonucleotides that bind to a specific miRNA of interest, thus blocking its function and regulating the post-transcriptional regulators [143,144]. miRNA mimics, synthetically produced and modified miRNAs that appear to block miRNA loading into Dicer, may be used in the future to block specific miRNAs from functioning in specific tumors. Mimics of let-7 and miR-34 have been tried in murine models of lung cancer, leading to reduction in the volume of the tumors [145,146]. It is not just about delivery of the mimics, though, but the delivery to the specific cell of interest. A few miRNA “drugs” have even been started in clinical trials and time will tell if miRNA treatments will be the wave of the future [147–149], but there is a lot of work to be done to transition our miRNA knowledge from the bench to bedside. The effectiveness and safety of miRNAs are of the utmost importance as these small molecules may be ideal as targeted forms of medicine. Personalized, non-invasive profiling and comparisons to the numerous miRNA signatures, may be the future of diagnostic medicine. Although the field has uncovered numerous miRNAs reported to be important in several aspects of cellular signaling and regulation in disease, the ability for miRNAs to be effectively used as diagnostic tools is still under investigation.

With the current, exciting technological advances in sequencing and array technologies, we can explore diseases with increasing depth of knowledge. It is more important than ever that the field does not become weighed down with seemingly endless big data sets without proper validation. This validation, such as via qPCR analysis, is not only important in correlating disease status to findings in an array, but becomes the first step in dissecting the targets and pathways in which these particular miRNAs function. In order to begin to delve into the seemingly endless lists of data, a wide array of publically available tools for *in silico* miRNA–mRNA and miRNA–pathway interactions have been developed (such as miRTARBASE, DIANA–miRPATH, miRWALK) [150–152]. With these tools, we may soon be able to not only create lists of miRNAs in disease, but profiles of the disease complete with regulators and targets. With many different large scale studies being performed on various cancer types, compiling data can be an additional challenge. Recently, a large meta-analysis study was performed by Wang et al. to compare over 100 studies on circulating miRNA profiles in blood serum and plasma in gastrointestinal cancer [153]. Studies such as this, are invaluable to future researchers to better choose and evaluate potential biomarkers and, furthermore, to begin to understand the pathways that they regulate. Additionally, many groups have utilized machine learning and have created training set of miRNAs in the hopes that these sets can be used in the future to simplify the prediction of disease [120,137,138]. Although this is not a trivial feat as one miRNA species can regulate numerous mRNAs, it is critical aspect in our understanding of disease and pushing the

field into the future.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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