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MicroRNA regulation of allergic inflammation and asthma

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Allergic diseases are prevalent and clinically heterogeneous, and are the pathologic consequence of inappropriate or exaggerated type 2 immune responses. In this review, we explore the role of microRNAs (miRNAs) in regulating allergic inflammation. We discuss how miRNAs, acting through target genes to modulate gene expression networks, impact multiple facets of immune cell function critical for type 2 immune responses including cell survival, proliferation, differentiation, and effector functions. Human and mouse studies indicate that miRNAs are significant regulators of allergic immune responses. Finally, investigations of extracellular miRNAs offer promise for noninvasive biomarkers and therapeutics strategies for allergy and asthma.

The challenge of allergy

In humans, robust type 2 immune responses are elicited by parasitic worm infections, insect bites and toxin exposure [1,2]. A role for type 2 immune cells and pathways has also emerged in tissue homeostasis, including the regulation of metabolism and wound healing. The pathologic consequences of exaggerated type 2 immune responses, often to apparently innocuous environmental stimuli, are prevalent and debilitating due chronic symptomatology. The resulting group of allergic diseases affects more than 10% of the population world wide and includes asthma, allergic rhinitis, atopic dermatitis, eosinophilic gastrointestinal disease, IgE-mediated anaphylaxis, as well as allergic responses to foods, contact agents and medications [3].

Allergic responses involve all major barrier tissues including the skin, nasal mucosa, lungs, and gastrointestinal tract, and occur in response to a diversity of inciting agents. The molecular and cellular networks that participate in type 2 immune responses are also complex, making the study of allergy a challenge. Multiple innate and adaptive immune cell types including eosinophils, mast cells, basophils, type 2 innate lymphoid cells (ILC2),

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alternatively activated macrophage (AAM), T helper (Th)2 cells, Th9 cells, T regulatory (Treg) cells and B cells, orchestrate and affect the response [4,5]. Non-hematopoietic cells, including glandular and non-glandular epithelium as well as smooth muscle, are essential for initiating the response and for end-organ changes that define disease. Critical factors include the production of IgE and cytokines such as thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, IL-33, IL-4, IL-13, IL-5 and IL-9.

Challenges that remain in allergy include understanding the inciting events, identifying critical regulatory nodes, defining cellular interactions that drive the response, and developing novel therapeutic strategies for treatment. Although investigations of how miRNAs and their target gene networks regulate allergic inflammation are still in their infancy, it is already clear that miRNAs have robust effects on immune responses and that their study can be used to address fundamental questions about type 2 immunity. Continued work is especially needed to characterize and define the cellular and molecular mechanisms by which miRNAs regulate allergy and asthma, to both enhance our basic understanding as well as leverage miRNA biology to address specific challenges in the prevention and treatment of these diseases.

MicroRNAs are dynamic post-transcriptional regulators of gene networks

miRNAs are small endogenous RNAs that regulate gene expression. They are transcribed from intergenic or intronic genomic loci into primary miRNAs (pri-miRNAs), often in polycistronic clusters. Pri-miRNAs are then sequentially processed into ~60 nucleotide precursor miRNAs (pre-miRNAs) and ~22 nucleotide mature miRNAs which are loaded into the miRNA-induced silencing complex (miRISC), which inhibits target gene expression by mRNA degradation or translational repression [6]. miRNAs identify targets for repression by imperfect base pairing to mRNAs, with the miRNA “seed sequence” (nucleotides 2-8) guiding target recognition. A single miRNA targets tens to hundreds of distinct mRNAs, and an individual mRNA may be directly regulated by multiple miRNAs. This results in large gene networks that may have robust effects on biologic processes, even with modest quantitative inhibition of individual miRNA-mRNA interactions [7]. Identification of critical miRNAs and elucidation of their targets will both enhance our understanding of the regulation of key determinants of allergic immune responses as well as offer the opportunity to identify novel genes and pathways that regulate allergy.

Single nucleotide polymorphisms in both miRNAs and miRNA target sites have been specifically linked to asthma, implicating miRNA activity directly in the pathogenesis of human allergic diseases. A polymorphism in pre-miR-146a that reduces mature miR-146a expression, likely through changes in nuclear processing [8], is associated with reduced asthma risk in both Chinese and Mexican patient cohorts [9,10]. Polymorphisms which introduce a new functional miR-148/miR-152 seed binding site in the 3′UTR of the non-classical immunomodulatory class I HLA gene, HLAG, or a mir-124 site for the integrin ITGB3 both confer protection from asthma [11,12]. Furthermore, the additional miRNA target site in HLAG correlates with reduced expression of soluble HLAG in bronchial lavage (BAL) from asthmatic subjects, consistent with enhanced miRNA-mediated gene repression [13]. Further work in model systems will help to quantitate and define how

modulation of miRNA levels and specific miRNA-target interactions affects key determinants of allergic responses.

Profiling miRNA expression in allergic inflammation

One approach to identify miRNAs involved in the pathogenesis of allergy is to uncover miRNAs that are differentially expressed in normal and affected tissue. Profiling studies of miRNAs in human biopsy specimens and mouse models of diseases including asthma, eosinophilic esophagitis and contact dermatitis show differential expression in ~10–20% of miRNAs. These studies have identified a group of shared miRNAs with altered expression in bulk lesional tissue and include let-7c, miR-21, miR-29, miR-135, miR-142, miR-146, miR-150, miR-155, miR-181, miR-193, miR-223, miR-365, miR-375, miR-452 and miR-615 [14–19]. Given the diversity of tissue sites and allergen exposures examined, the identification of these miRNAs points to shared cellular and molecular components of a pathologic type 2 immune response.

These profiling results likely reflect changes in the cellular composition of the tissue, as allergic responses are characterized by both the influx of inflammatory cells as well as reactive epithelial and stromal changes. For example, several of these miRNAs including miR-21, miR-135a, miR-146b, miR-193b and miR-223 are upregulated during *in vitro* differentiation of eosinophils [20–23]. Therefore, preferential expression in allergic tissues may reflect recruitment of these cells, a hallmark of type 2 immune responses. Indeed, correlation between cell recruitment and miRNA expression has been specifically demonstrated for $CD4^+$ T cells infiltrating the skin in atopic dermatitis, which provide the major cellular source of miR-155 in lesional tissue [18]. Further studies focusing on differential miRNA expression in relevant isolated or sorted cell populations has provided a means to focus on candidate miRNA with functional relevance in allergy [24,25]. Testing of individual miRNAs in model systems of allergy and asthma has led to the identification and characterization of miRNAs involved in pathogenic type 2 immune responses.

miRNAs in survival, production, and proliferation of type 2 immune cells

Any miRNA that impacts the homeostatic functions in cells that are essential for a type 2 immune response may positively or negatively regulate allergy. Although numerous miRNAs affect T cell, B cell and myeloid cell activation, survival, and proliferation [26–28], less is known about miRNA regulation of many of the innate cell subsets important for allergy, particularly ILC2 and basophils. In eosinophils, miR-21 contributes to and miR-223 limits cell production, survival and proliferation [22,23]. Mechanistic investigations suggest that miR-223 may regulate eosinophil proliferation in part through targeting a growth factor receptor, IGFR2 [23]. In mast cells, miR-221/222 are upregulated upon activation and inhibit cell cycle [29]. Further investigations are needed to determine what impacts these effects may have on allergic disease and identify groups of essential downstream target genes through which they act (Figure 1a).

miRNAs in the differentiation and polarization of cells of the type 2 immune response

Critical to the propagation of allergic inflammation is the expression of specific effector gene programs required for a type 2 immune response. miRNAs regulate this differentiation process, often by acting directly on the expression of key transcription factors (Figure 1b). For example, the polarization of macrophages to the “M2” phenotype characteristic of type 2 responses is regulated by miRNAs. Addition of IL-4 or IL-13 induces expression of miRNAs including miR-124 and miR-223 in macrophage cultures, and both contribute to M2 polarization [30,31]. *In vivo*, miR-124 is also upregulated in lung macrophages in allergic airway inflammation models [30]. Both of these miRNAs directly target transcription factors to influence macrophage differentiation, with miR-124 targeting C/EBP-α and miR-223 targeting Pknox1, a novel regulator of M2 lineage polarization [31,32].

The differentiation of naïve $CD4^+$ T cells into Th2 cells to produce cytokines including IL-4, IL-13 and IL-5 are central events in allergy. Overexpression of miR-21 promotes the *in vitro* differentiation of Th2 cells, while miR-27 and miR-128 inhibit IL-4 and IL-5 production in activated $CD4^+$ T cells [33,34]. miR-155 is upregulated in human $CCR4^+$ Th2-enriched $CD4^+$ T cell subsets, inhibits Th2 cell differentiation and cytokine production *in vitro*, and directly targets the IL-4 transactivating transcription factor *Maf* [25,35,36]. Helper T cell differentiation may be particularly sensitive to miRNA regulation due to cytokine and transcription factor mediated positive feedback loops that amplify small perturbations in extracellular signals and intracellular signal transduction into large effects on gene expression programs and cell identity.

miRNAs participate in gene networks that regulate signaling pathways in type 2 effector responses

The principle that miRNAs target multiple mRNAs to form regulatory networks is evident in type 2 immune responses. Studies in multiple cell types, diseases and model systems have shown that individual miRNAs can positively or negatively regulate allergic responses, often through the modulation of key signaling pathways (Figure 1c). miR-19a is upregulated in airway infiltrating T cells from asthmatic patients and promotes Th2 cell cytokine production in *in vitro* differentiation assays, acting on the mRNAs that encode PTEN, SOCS1 and A20 to coordinately de-repress several signaling pathways [37]. miR-146a is upregulated in the keratinocytes of patients with atopic dermatitis and inhibits numerous IFN-γ inducible and atopic dermatitis-associated genes [38]. Broader changes in gene expression networks coordinate with effects on direct targets that regulate upstream NF-κB signaling, including IRAK1 and CARD10, and downstream effector genes, including CCL5, to guide inflammatory cell recruitment.

In the case of FcεR-mediated mast cell functions, multiple miRNAs converge to regulate common signaling pathways. Mast cell degranulation and cytokine production are inhibited by both miR-155 and miR-223 and correlate with selective alterations in PI3K-AKT pathway activity, though the direct mRNA targets remain to be identified [39,40].

Degranulation and adherence in response to FcεR ligation are enhanced by both miR-142-3p and miR-221. miR-142-3p directly targets LPP, which regulates actin and inhibits degranulation in mast cell lines [41], and miR-221 induced changes in cytoskeletal gene expression in transduced mast cells [42], suggesting that these miRNAs regulate shared downstream pathways. Important challenges remain to identify the molecular networks through which these various miRNAs act, and to understand how the control they exert is integrated in the context of an allergic response.

Investigations of miRNAs in epithelial and smooth muscle cells has also demonstrated a role for miRNAs in regulating non-immune cell signaling pathways involved in allergic inflammatory responses. Smooth muscle and epithelial proliferation are hallmarks of tissue remolding in asthma. miRNA regulation of TGF-β signaling has been implicated in both of these cell types. miR-221 expression is more highly induced by TGF-β in airway smooth muscle cells in patients with severe asthma compared with healthy controls, and miR-221 promotes proliferation and IL-6 secretion [43]. miR-19a is upregulated in the bronchial epithelium of severe asthmatics and also enhances proliferation [44]. miR-19a directly targets TGFβR2, and overexpression or inhibition of this miRNA is associated with changes in downstream SMAD3 signaling. Inhibition of PI3K-AKT-CDK signaling in human airway smooth muscle cells by miR-10a inhibits smooth muscle proliferation, and mir-10a directly targets the mRNA of the catalytic subunit PIK3CA [45]. Hyperstretch can also contribute to the pathogenesis of obstructive lung diseases, such as allergic asthma. miR-155 is induced by stretch in human bronchial epithelium, contributes to IL-8 secretion, and directly targets the phosphatase SHIP1[46]. Taken together, these studies reinforce the paradigm that miRNAs regulate cell responses and function by inhibiting the expression of target gene networks. However, the identity of the key, limiting target genes (even for the same miRNA) vary in different cell types and contexts.

miRNA in the treatment of allergic disease

Experiments in mouse model systems of allergy and asthma have demonstrated that individual miRNAs can significantly regulate pathogenic type 2 immune responses (Figure 2a). miR-155$^{-/-}$ mice have reduce airway hypersensitivity and increased passive cutaneous anaphylaxis responses [39,47]. T cell-intrinsic expression of miR-155 promotes airway hyperresponsiveness (AHR) in asthma models, in part through the regulation of the direct target S1pr1 and recruitment of effector cells to the lung [47,48]. miR-21$^{-/-}$ mice also have reduced allergic inflammation in the lung after allergen challenge, with a shift toward Th1 differentiation and increased dendritic cell IL-12 and T cell IFN-γ production [49].

miRNA-directed therapeutics for allergic diseases are an attractive area of investigation for several reasons. Clearly, miRNAs can impact *in vivo* allergic responses, and agents that modulate miRNA activity are easy to design and construct using base pairing chemistry. Moreover, allergic responses occur at accessible barrier surfaces, circumventing some of the challenges to delivery of nucleic acid-based therapeutics., Experiments in mouse models that dose miRNA mimics or inhibitors for let-7a, mir-106a, miR-126, mir-221 and mir-145 suggest that pharmacologic manipulation of miRNA activity is capable of altering airway inflammation and/or AHR [19,50–54]. miRNA-directed therapeutics may even someday

offer specific treatments for disease subtypes. miR-9 antagonists restore dexamethasone sensitivity in models of steroid-resistant AHR [55]. For each of these miRNAs, further work is needed to determine how altering miRNA activity *in vivo* can alter allergic responses, including which cell types and target pathways are responsible for the observed responses.

However, an inability to direct delivery and assess activity in relevant cell types *in vivo* remain major challenges in the field, and are compounded by the pleiotropic effects of miRNAs. In some cases, *in vivo* results and known miRNA-target interactions are well correlated. miR-9 expression is increased in lung macrophages in steroid-resistant airway hypersensitivity models, directly targets regulatory subunits of protein phosphatase 2A, and alters glucocorticoid signaling consistent with miR-9 antagonists ameliorating steroid-resistant AHR [55]. However, in other studies, discordance between molecular, *in vitro* and *in vivo* data have been observed. Although let-7 family members directly target the 3′UTR of IL-13, effects of let-7 inhibitors and mimics *in vivo* have given contradictory results in mouse asthma models [19,50,51]. This likely reflects the fact that miRNA activity depends on an integrated effect on direct mRNA targets expressed in a single cell type, activity in multiple cell types, as well as the hierarchical importance of these factors in mounting an effective allergic response.

Extracellular miRNAs

Although miRNAs have largely been studied for their cell-intrinsic roles, these small RNAs are both present and stable in a diverse array of extracellular body fluids including blood serum/plasma, BAL, saliva, peritoneal fluid, pleural fluid, cerebrospinal fluid and urine [56]. Extracellular miRNAs (ex-miRNAs) exist in different forms, including within nanovesicles generated from multivesicular bodies termed exosomes, within lipoprotein complexes, and bound to Argonaute proteins outside of vesicles [57–59] (Figure 2b). Functional ex-miRNAs can be secreted and transferred between dendritic cells, from macrophages to epithelial cell lines, and between T cells and antigen presenting cells, at least *in vitro* and possibly *in vivo* as well [60–64]. Together these findings suggest that ex-miRNA might be useful disease biomarkers, and that they may even constitute a novel form of immune cell communication that could be exploited for therapeutic RNA delivery.

Indeed, preliminary studies suggest that ex-miRNAs may be useful as biomarkers for allergic disease, with the ability to classify disease subtype or activity, and that biologically relevant extracellular miRNAs may contribute to the pathogenesis of allergic disease. Profiling of exosomes in BAL has revealed significant differences in miRNA expression between asthmatic patients and controls, with correlations to lung function and atopy [65]. Hundreds of miRNAs, apparently within exosomes, can even be detected after collection of exhaled breath condensate and could provide noninvasive diagnostic tools for allergic disease in the lung [66,67]. Investigations into extracellular miRNAs may ultimately even produce novel therapeutic strategies, as antigen-specific exosomes through the delivery of miR-150 are capable of inhibiting allergic contact hypersensitivity responses in mice [68].

Conclusions

miRNAs are important post-transcriptional regulators of gene expression and have a role in allergic type 2 immune responses through their activity in multiple immune and non-immune cell subsets. Detailed mechanistic studies are critically needed to understand and leverage miRNAs to advance the field and inform clinical investigation. miRNAs act through multiple direct targets to regulate networks of genes, and their specificity and potency depends on the dynamics of individual miRNA-target interactions. Identifying which miRNAs and which targets are important for promoting or restraining allergy will help to identify vulnerable nodes in allergic inflammation, enhancing our mechanistic understanding of miRNA in the immune system and providing novel, possibly druggable, targets for these increasingly prevalent diseases.

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Highlights

- miRNAs regulate allergic inflammation and allergic responses.
- miRNAs act coordinately through target gene networks.
- miRNAs impact diverse cellular functions in type 2 immune cells.
- miRNAs provide novel biomarkers and therapeutic strategies in allergy.

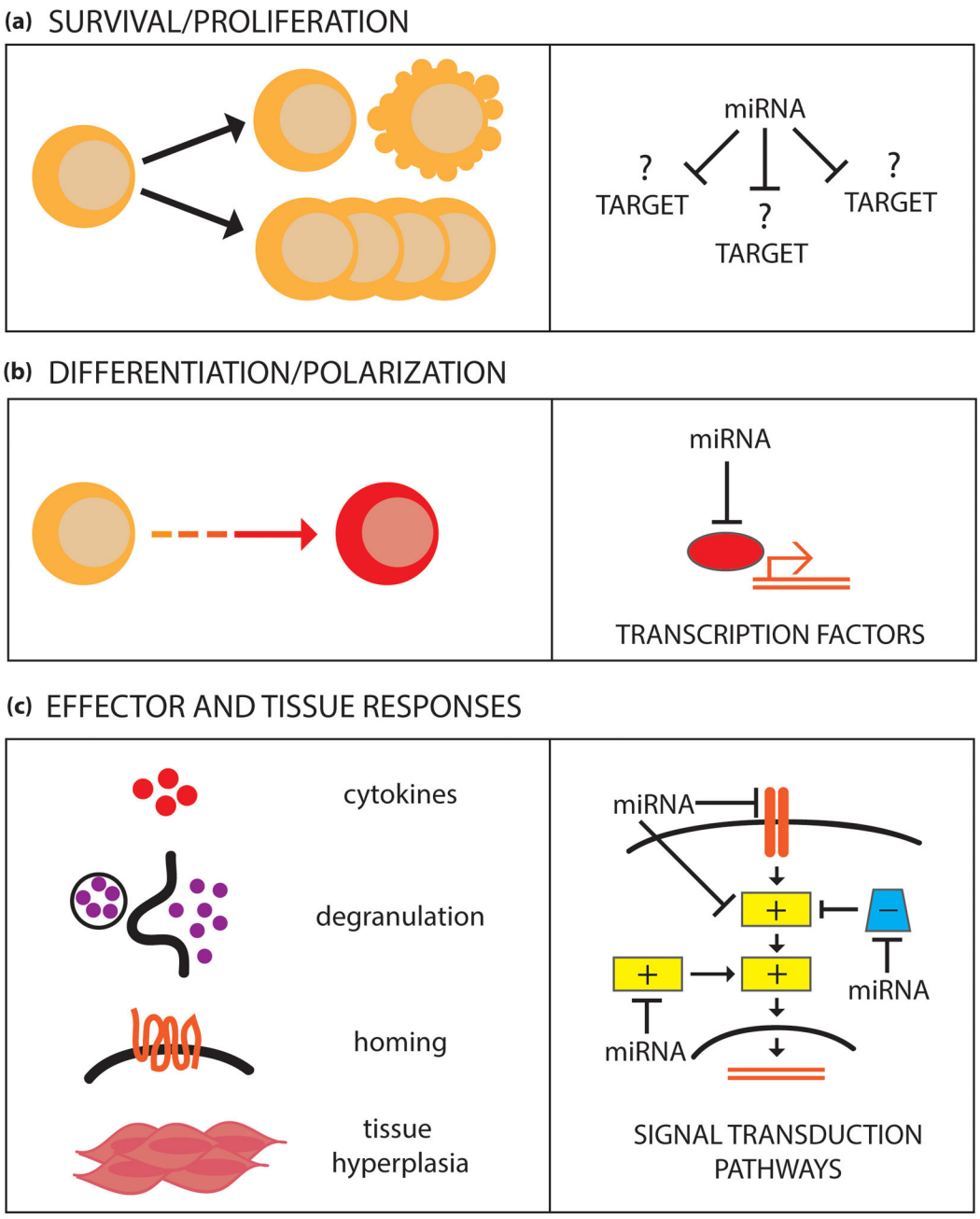

Figure 1.

miRNAs regulate multiple facets of type 2 cell function. They act in diverse cellular processes from **(a)** survival/proliferation to **(b)** differentiation/polarization to **(c)** effector and tissue responses. This enables miRNAs to have robust effects on allergic immune responses. Although much work remains to identify and understand the target gene networks through which miRNAs act, common themes have emerged including miRNA regulation of **(b)** transcription factors in differentiation/polarization and **(c)** signal transduction pathways in effector responses. Continued investigations offer the opportunity to both expand our

understanding of how miRNAs act through multiple downstream targets to regulate immune responses and also identify novel pathways important for allergic inflammation.

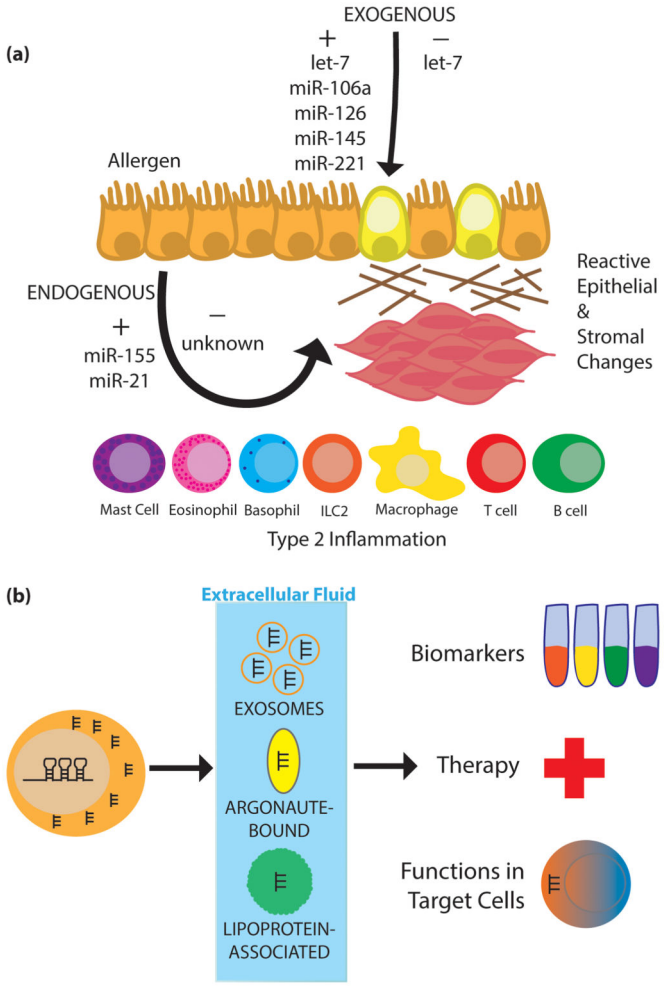

Figure 2.

miRNAs as endogenous regulators, novel biomarkers and potential therapeutic agents in allergic inflammation. **(a)** Genetic loss of function studies and administration of miRNA mimics/inhibitors demonstrate a significant role for the endogenous expression and exogenous manipulation of individual miRNAs in the regulation of mouse models of asthma. **(b)** Extracellular miRNAs are stable and present in body fluids within exosomes, bound to Argonaute and associated with lipoproteins. While their cellular sources and potential functions remain largely unknown, they have the potential to guide the development of novel biomarkers and therapies in diseases including allergy and asthma.