ncRNA regulated immune response and its role in inflammatory lung diseases

Na Xie, Gang Liu

Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine,
University of Alabama at Birmingham, Birmingham, AL 35294

Address correspondence to:

Gang Liu, M.D., Ph.D.
Associate Professor
Department of Medicine
University of Alabama at Birmingham
901 19th St. So., BMR II 233
Birmingham, Alabama 35294
Tel: 205-975-8932
Fax: 205-934-7437
E-mail: gliu@uab.edu

Keywords: Non-coding RNA; immunity; asthma; COPD; cystic fibrosis, ARDS
Abstract

Despite the greatly expanded knowledge on the regulation of immune response by protein molecules, there is increasing understanding that non-coding RNAs (ncRNAs) are also an integral component of this regulatory network. Abnormal immune response serves a central role in the initiation, progression and exacerbation of inflammatory lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and acute respiratory distress syndrome (ARDS)/acute lung injury (ALI). Dysregulation of ncRNAs has been linked to various immunopathologies. In this review, we highlighted the role of ncRNAs in the regulation of innate and adaptive immunity, as well as summarized recent findings that ncRNAs participate in the pathogenesis of inflammatory lung diseases via their regulation of pulmonary immunity. We also discussed therapeutic potentials for targeting ncRNAs to treat these lung disorders.
Introduction

Pathogenesis of inflammatory lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and acute respiratory distress syndrome (ARDS)/acute lung injury (ALI), involves numerous molecular and cellular mechanisms. However, dysregulated immune response has been recognized as a quintessential event that plays a critical role in the initiation, progression and exacerbation of these lung diseases (5, 36). Understanding the immunological paradigm in these disorders will clarify the mechanisms underlying the persistent inflammation and failed immune resolution associated with these conditions and will help to design novel therapeutics that target disease specific immune dysregulations.

Innate immune response is the first line of host defense against microbial organisms and other deleterious exogenous or endogenous pathogens (39, 42, 68, 100). These agents are presented in the form of pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs), which can be recognized by surface or intracellular pattern recognition receptors (PRRs) of the myeloid derived immune cells, including granulocytes, monocytes, macrophages, and dendritic cells (DCs), as well as non-myeloid derived cells (11, 100). Engagement of PAMPs or DAMPs with specific receptors activates a signaling cascade that drives these cells to produce pro-inflammatory cytokines, chemokines, reactive oxygen and nitrogen species, and antimicrobial peptides, as well as boosts phagocytic activity of the cells (39, 42, 68, 100). Innate immunity is essential for the eradication of infections and rapid removal of unhealthy or deceased cells (39, 42, 68, 100). Additionally, an appropriate innate immune response is also critical to initiate and shape specific adaptive immunity that encompasses T and B lymphocytes (69).

Despite the benefits of inflammation, hyperactive and/or unresolving immune responses can cause tissue pathology (77). Therefore, the cellular and molecular networks that control the initiation, magnitude, and resolution of inflammation must be tightly regulated to achieve the balance between the optimization of host response and the homeostasis of immune system (77). Although our understanding of immune response at the transcriptional and epigenetic
levels has been well developed due to several decades of collective efforts (1, 95), an emerging theme in recent years is the central role of noncoding RNAs (ncRNAs) in the regulation of this dynamic process. ncRNAs have been shown to participate in lineage differentiation, proliferation, activation and function of various types of immune cells. More pertinently, deregulation in ncRNA expression was frequently found to accompany diseases associated with excessive or uncontrolled inflammation (23, 26, 77, 79, 90).

The biology of ncRNAs

Recent advance in transcriptome analysis demonstrated that active transcription from mammalian genome was more pervasive than we had ever anticipated. However, most of the RNA transcripts are non-coding (25, 64, 98). Except ncRNAs that participate in protein synthesis, RNA splicing, and nuclear organization, including transfer RNAs (tRNAs), ribosome RNAs (rRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), a large fraction of these ncRNAs have important regulatory roles in various biological processes and are therefore regarded as regulatory ncRNAs, such as microRNAs (miRNAs) and long ncRNAs (lncRNAs) (16, 25, 109).

miRNAs are primarily transcribed through RNA polymerase II (6, 10, 13, 122). The primary transcripts (pri-miRNA) are processed by RNase III endoribonuclease Drosha in nucleus to give rise to precursor miRNAs (pre-miRNA), which are further excised by endoribonuclease Dicer in cytoplasm to generate ~22 bp mature miRNA (6, 16, 44, 122). To operate, the “seed” sequence (n.t. 2-8 from the 5’ end) of the miRNAs serves as a guide for the microRNA-induced silencing complex (miRISC) to recognize the three prime untranslated region (3’ UTR) of target mRNAs through Watson-Crick base-pairing. After binding, miRNAs either promote mRNA degradation or repress mRNA translation, and thereby they inhibit the expression of target proteins. Recent evidence additionally showed that some miRNAs can activate cells by binding to Toll like receptors (54). This non-canonical activity of miRNAs adds an additional complexity to the studies of miRNA functions. LncRNAs generally refer to ncRNAs larger than 200 bp. LncRNAs do not possess protein-coding capability but function primarily through
interactions with proteins, DNAs or RNAs (109). As a result, lncRNAs affect gene expression at the epigenetic, translational and posttranslational levels (109).

Not only are not all miRNAs evolutionally conserved, but the 3' UTR sequence of a homologous gene can also vary across species. Therefore, the very same miRNA may target distinct sets of genes in different organisms so that functions of a specific miRNA demonstrated in animals may not faithfully reflect its role in human. In addition, one may not be able to find suitable animal models to test the activity of human specific miRNAs. LncRNAs display much less sequence conservation among species (109), which similarly renders it technically challenging to determine the role of human specific lncRNAs in vivo.

ncRNAs and innate immunity

miRNAs regulate innate immune response. The role of miRNAs in innate immunity was initially indicated after discovering altered miRNA expression profile in stimulated immune cells (11, 99). To date, there are more than two dozens of individual miRNAs implicated in modulating this process. Despite the involvement of multiple miRNAs and their effect on different innate immune cells, the central characteristic of such a regulation is that these miRNAs target key mediators in the molecular network of innate immunity, ranging from pattern recognition receptors and kinase regulators to transcriptional factors and epigenetic modifiers. In one of the pioneering studies from the field, Baltimore’s group demonstrated that miR-146a was rapidly upregulated in human monocytic cells treated with lipopolysaccharide (LPS) and acted in a negative feedback mechanism to dampen inflammatory response, probably by targeting tumor necrosis factor receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) (99). In following studies, miR-146a has been shown to play negative roles in various inflammatory settings, such as its regulation of endotoxin tolerance and responses to bacterial and viral infections (15, 95).

Like miR-146, both miR-21 and miR-155 can be induced by LPS stimulation (1, 92). However, they have antagonistic functions in innate immune response. miR-21 was discovered to inhibit LPS induced NF-κB activation and IL-6 expression by targeting tumor suppressor
PDCD4, a pro-inflammatory protein (92), whereas miR-155 is pro-inflammatory, acting by repressing negative regulators of inflammation, such as suppressor of cytokine signaling 1 (SOCS1) and phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1) (3, 75). This raises an intriguing question regarding why inflammatory cells need two miRNAs with opposing activities while responding to LPS. A plausible explanation is that miR-155 may rise earlier than miR-21 in order to mobilize a maximum immune response to pathogen invasion, which later needs to be dampened by miR-21 to return the immune program to homeostasis (66). Such a phenomenon does represent a good example that multiple miRNAs can act in coordination to fine tune inflammatory responses.

Of all myeloid innate immune cells, macrophages are evidently a unique group of cells in that they display phenotypic continuum, with plasticity to sway between two opposite extremes, namely the M1 and the M2 states (54). Macrophages at the two polarized states have vastly different functions in innate immune response as well as in adaptive immunity (54). Macrophages that are activated by INF-γ and/or various TLR agonists are M1 macrophages. They participate in clearance of bacterial, viral and fungal infections as well as immunosurveillance of tumors (50, 70, 93). M2 macrophages are induced by IL-4/IL-13, immune complex plus TLR ligands, IL-10, TGF-β1, or glycocorticoids and are involved in feedback to parasite infection, tissue remodeling, tumor progression (62, 74, 114), and immunomodulation (2, 60, 62). The role of miRNAs in macrophage polarization has been well established. Since M1 macrophages are in general pro-inflammatory, miRNAs that are involved in innate immunity can presumably regulate M1 macrophage activation. The regulatory activity of many of these miRNAs in innate immune response was indeed discovered in macrophages, including anti-M1 miRNAs, such as miR-146a, miR-147, and miR-21, and pro-M1 miRNAs, such as miR-155, miR-125b, miR-27a, and miR-27b (18, 40, 55, 78, 92, 99, 118). Similar to the way in which they function in M1 macrophages, miRNAs modulate M2 macrophage activation primarily through targeting key M2 mediators. Studies from our group and others demonstrated that miR-124, miR-223, miR-125a-5p and let-7c are positive regulators of the M2 macrophage polarization (4, 107, 125). Interestingly, all of these miRNAs were also capable of suppressing M1 macrophage activation (4, 85, 125). These findings indicate that miRNAs are critical modulators of macrophage plasticity.
**LncRNAs regulate innate immune response.** Although the functional importance of miRNAs in innate immunity has been established, the role of LncRNAs in this process is comparatively less defined (90). The first in-depth study on this subject came from Carpenter et al. (14). They performed a whole transcriptome analysis (RNA-seq) on macrophages treated with the synthetic TLR2 ligand Pam3CSK4 and identified a number of TLR agonist induced LncRNAs (14). One of these LncRNAs, lincRNA-Cox2, was demonstrated to participate in the activation and repression of immune response genes through its interactions with multiple heterogeneous nuclear ribonucleoproteins (hnRNPs) (14). In a similar fashion, the long noncoding RNA THRIL controlled TNF-α expression via its interaction with hnRNPL (53). Altogether, these studies suggest that many LncRNAs use common mechanisms to regulate innate immunity. However, Imamura et al. showed a different way in which LncRNAs modulate inflammatory response (37). They found that the LncRNA nuclear enriched abundant transcript 1 (NEAT1) promoted IL-8 production by relocating splicing factor proline/glutamine-rich (SFPQ), a NEAT1-binding paraspeckle protein as well as a repressor of IL-8 transcription, from the IL-8 promoter to the paraspeckles, and thereby de-repressing IL-8 expression (37). In our own studies, we performed a profiling on LncRNA expression and identified a number of LncRNAs that displayed altered expression in LPS treated monocytes (24). We characterized one of the LPS induced LncRNAs, Lnc-IL7R, which overlapped with the 3' UTR of the human IL-7 receptor α-subunit gene (*IL7R*). We found that Lnc-IL7R inhibited inflammatory response presumably by inducing trimethylation of histone H3 at lysine 27 (H3K27me3), a hallmark of inactive chromatin, at the proximal promoter regions of inflammatory mediators (24).

**ncRNAs and adaptive immunity**

Adaptive immune response is executed by antigen-specific T and B cells. It has been established that a dynamic transcription-regulated proteome controls the development, differentiation, activation and functions of lymphocytes to provide specific host defense against subsequent exposure to the same pathogen (124). There is also increasing appreciation that ncRNAs are indispensable regulatory elements in these processes (7, 30).
ncRNA regulated development, differentiation, and function of T cells. The functional necessity of miRNAs in T cell development and activation was initially recognized after observing that mice with Dicer deletion experienced reduced T cell numbers in thymus and peripheral lymphoid organs as well as aberrant T helper (Th) cell differentiation and cytokine production (20, 71). Following lead, later studies identified individual miRNAs that played critical roles in T cell biology. miR-155 is representative in such a regulatory paradigm. T cells deficient in miR-155 displayed a Th2 phenotype under neutral conditions in vitro (87, 102). Furthermore, miR-155-/- mice were defective in Th1 and Th17 cell production, which led to increased resistance to experimental autoimmune encephalomyelitis, colitis, and collagen induced arthritis in these mice (49, 73, 76, 80). The pro-Th1 activity of miR-155 is in accordance with its pro-inflammatory role in innate immune response (75). However, miR-155 targeted a completely different set of mediators in T cells from those in macrophages and monocytes. Taken together, these studies suggest that a single miRNA can act on multiple elements of the immune system to achieve a collective effect. miR-17-92 cluster miRNAs regulate T cell survival by repressing Bim and PTEN, both of which are pro-apoptotic (117). Overexpression of the miR-17-92 cluster in T cell populations led to increased proliferation and decreased apoptosis (117). miR-326 targeted Ets, a transcription factor that inhibits Th17 development (28). As a consequence, overexpression of miR-326 led to Th17 differentiation and IL-17 production (28). miRNAs are also involved in intrinsic regulation of T cell development. miR-181a was shown to control the sensitivity of T cells to peptide antigens (52). Increasing miR-181a expression in mature T cells enhanced the sensitivity to peptide antigens, while inhibiting miR-181a expression in the immature T cells decreased sensitivity and impaired positive and negative selection (52). These studies suggest that miR-181a functions as an intrinsic antigen sensitivity “rheostat” during T cell development. In addition to the regulation of effector T cells, miRNAs were also shown to participate in the development and function of T regulator (Treg) cells. For instance, miR-155-/- deficiency led to reduced numbers of Tregs in the thymus and peripheral lymphoid tissues, whereas it had no effect on the suppressive activity of Tregs (45). In contrast, miR-146a-/- Treg cells were defective in suppressing Th cell activity (56). miR-146a deletion in Treg cells led to breakdown of immunological tolerance caused by IFN-γ-dependent immunopathology in multiple organs (56). Collectively, these studies propose that versatile mechanisms are employed by miRNAs
in the regulation of T cell biology. Therefore, dysregulations of such miRNA controlled events could lead to aberrant adaptive immune response and immunopathology.

In addition to miRNAs, recent in vivo studies also lent strong support for the functional significance of lncRNAs in adaptive immunity (90). One of the initial studies identified hundreds of lncRNAs in CD8+ T cells, many of which were lymphoid-specific and/or underwent altered expression during lymphocyte differentiation or activation (83). A number of these lncRNAs are physically close to immunologically important genes and were predicted to function via a range of regulatory mechanisms (83). Gomez et al. found that an enhancer-like lncRNA termed NeST (nettoie Salmonella pas Theiler’s) was responsible for the susceptibility of SJL/J mice to persistent infection by Theiler’s virus (31). The NeST gene is located in the locus Tmevp3 (Theiler’s Murine Encephalitis Virus Possible 3), which also contains IFN-γ (31). Further studies showed that transgenic expression of NeST increased Theiler’s virus persistence and decreased Salmonella enterica pathogenesis (31). NeST promoted the expression of IFN-γ in activated CD8+ T cells through interaction with WDR5, a component of the histone H3 lysine 4 methyltransferase complexes, and thereby enhancing histone H3 methylation at the IFN-γ locus (31). Similarly, lncRNA-CD244 was shown to recruit polycomb protein enhancer of zeste homolog 2 (EZH2) to the IFN-γ and TNF-α promoters in CD8+ T cells (111). Such an epigenetic mechanism was also involved in lncRNA regulation of CD4+ T cell differentiation. Ranzani et. al recently showed that linc-MAF-4, a chromatin-associated lncRNA specific to the Th1 subsets, inhibited the expression of MAF, a Th2 transcription factor, by recruiting the chromatin modifiers lysine-specific demethylase 1 (LSD1) and EZH2 to the MAF promoter (86). LncRNAs regulate adaptive immunity not only at the transcriptional levels, but also by modulating protein modification and translocation. One such example is the finding involving lncRNA NRON (non-protein-coding RNA, repressor of NFAT) (91). NRON repressed the transcription of genes activated by NFAT (nuclear factor of activated T cells), a calcium-dependent transcription factor that is critical for T cell activation (91). Further studies discovered that NRON formed a large RNA-protein complex that sequestered phosphorylated NFAT in the cytoplasm in resting T cells (91). This complex also contained the calmodulin-binding protein IQGAP1 (IQ-motif-containing GTPase-activating protein 1) and nuclear transport factors, including KPNB1 (karyopherin beta 1) (91).
Knockdown of NRON led to increased dephosphorylation and nuclear translocation of NFAT and cytokine production in activated T cells (91). Collectively, these studies indicate that lncRNAs can exert their regulatory functions in T cells through interactions with diverse mediators. LncRNAs also regulate DC differentiation, and thereby they indirectly modulate T cell activation. This was shown by the study demonstrating that lncRNA Inc-DC promoted DC differentiation by activating STAT3 (110).

**ncRNA regulated development and function of B lymphocytes.** B cells are responsible for production of antigen specific antibodies during adaptive immune response (65). B cells develop in the primary lymphoid tissues and reach functional maturation in the secondary lymphoid tissues (65). It is widely accepted that a coordinated transcription program conducts a critical regulatory role in B cell development, maturation and activation (88). Recent evidence further demonstrated that multiple miRNAs participated in these cellular events. First of all, it was shown that conditional knockout of Dicer or Ago2 in early B cell progenitors almost completely blocked the transition of pro-B to pre-B cells, underscoring the essential function of miRNAs in B cell development (46). Concerning the role of individual miRNAs in B cell biology, the miR-17-92 cluster and miR-181a were shown to be positive regulators of B cell differentiation in that deletion of miR-17-92 impaired the transition from pro-B to pre-B cells and ectopic expression of miR-181 in hematopoietic stem cells led to increased B cell generation (19, 106). In contrast, ectopic expression of miR-150 in hematopoietic stem cells reduced the mature B cell population by targeting c-Myb, a key transcription factor in B cell development (116). The increasing expression of miR-150 during B cell maturation was inversely correlated to that of c-Myb, suggesting that this miRNA is required for normal B cell development (116). Additionally, B cells lacking miR-155 produced attenuated extrafollicular and germinal center responses and failed to generate high-affinity IgG1 antibodies (102).

**Immuno-ncRNAs and inflammatory lung diseases**

It has been increasingly clear that specific ncRNAs are essential for the development, differentiation, activation, and function of cells from the two arms of immune response. The temporal and spatial expression of ncRNAs in these cells needs to be tightly regulated in order
to produce an optimum immune response to foreign and endogenous pathogens. Dysregulation
of specific ncRNAs is likely leading to immunopathologies, including several lung diseases
that are characteristic of aberrant immune response (Figure 1).

**ncRNAs in immune regulation and asthma**

Asthma is a common chronic disorder characterized by airway inflammation accompanied by
airway hyperresponsiveness (AHR) and obstruction and pathological lung remodeling (22, 43). Allergic asthma is the most common form of asthma, which is normally triggered by inhaled allergens, such as pollen and house dust mite. Both innate and adaptive immune cells are responsible for the immunopathologies in asthma (43). However, hyperreactive Th2 immune response has been established as the predominant cause of inflammatory manifestations in this disease (43).

The early evidence that miRNAs participate in asthma pathogenesis through modulating Th2 cell function came with the study by Foster and colleagues (63). They found that selective blockade of miR-126 suppressed house dust mite induced asthmatic phenotype, including Th2 responses in mouse lungs (63). The study identified POU domain class 2 associating factor 1 as the mediator for the pro-asthmatic activity of miR-126 in that this protein activated the transcription factor PU.1 (63). PU.1 is known to inhibit Th2 cell function via downregulating the expression of GATA3, a critical positive transcriptional factor in Th2 activation. This study established the first causal link between miRNA expression and asthma pathogenesis and suggests that targeting miRNA in the airways may lead to anti-inflammatory treatments for allergic asthma. The critical role of PU.1-miRNA axis in Th2 cells was recently further confirmed by another study showing that miR-155 deficiency led to diminished eosinophilic inflammation and mucus hyper-secretion in the lungs of allergen treated mice (59). The implication of Th cells in this setting was confirmed by the finding that adoptive transfer of CD4⁺ T cells restored the airway eosinophilia in miR-155 KO mice to the degree similar to that found in wild type mice (59).
Given the overwhelming role of Th2 lymphocytes in asthma pathogenesis, there has been a systemic effort to determine how Th2 cells are regulated by miRNAs in regard to their activation, proliferation, survival, differentiation and function. A recent study performed a meticulous profiling on miRNA expression in human airway infiltrating T cells and discovered elevated expression of a miRNA, miR-19a, in asthma. miR-19a expression was increased in CD4+ T cells from asthmatic lungs (94). Deletion of the miR-17-92 cluster, which includes miR-19a, attenuated Th2-driven inflammation in vivo and Th2 cytokine expression in vitro. The defective Th2 response could be rescued by miR-19a and miR-19b, indicating a more prominent role of miR-19 among this miRNA cluster (94). Additionally, the pro-Th2 activity of miR-19 was shown to be cell intrinsic and proliferation independent. These authors also identified several targets that are known to negatively regulate Th2 cytokine production, including SOCS1, TNFAIP3, PTEN (94).

IL-13 is one of the primary Th2 cytokines and critically involved in inflammation and tissue remodeling in allergic asthma. Unsurprisingly, there were two groups that independently discovered that the miRNA let-7 family targeted IL-13 in mouse asthma models (47, 84). These findings indicate that let-7 could participate in asthma pathogenesis by directly regulating the functions of Th2 lymphocytes. Additional experiments showed that expression of most let-7 family members was inversely associated with the severity of allergic airway inflammation (47). However, experiments also found that both suppression and supplement of let-7 in mouse lungs ameliorated the asthmatic phenotype in mouse lungs (47, 84). These seemingly contradictory results underscore the intricate nature of miRNA targeting in vivo, particularly when a multi-member miRNA family is involved. Blocking one miRNA in a family could boost others' activity through Dicer negative feedback response (47, 48, 84). A better strategy could be targeting the "seed" sequence that is shared across the family. In fact, a tiny seed-targeting 8-mer locked nucleic acid (LNA) probes were recently shown to successfully block the miR-17–92 cluster family in vivo (72).

The anti-inflammatory miR-21 can tip off the balance between Th1 and Th2 responses to allergic antigens in asthma. Targeted ablation of miR-21 led to reduced lung eosinophilia in asthmatic lungs, but significantly increased levels of the Th1 cytokine IFN-γ (57). These data are in accordance with previous findings that IL-12p35 is a functional target of miR-21 and
miR-21 deficient DCs expressed more IL-12 upon LPS stimulation (57, 58). Additionally, OVA-challenged miR-21-/- CD4^+ T lymphocytes produced increased IFN-γ and decreased IL-4 (57). These data underscore the current understanding that Th1 associated cytokines, including IFN-γ, IL-12 and IL-18, inhibit type 2 immunity (115). Apparently, miR-21 conducts the pivotal role in Th2 mediated asthma by downregulating these Th1 cytokines.

miRNAs are not only involved in asthma pathogenesis, they can also dictate therapeutic responses to some therapies. It has been recently shown that miR-9 regulates steroid resistant AHR in asthmatic subjects (51). This effect was achieved presumably by miR-9 targeting of protein phosphatase 2 regulatory subunit B (B56) δ isoform, which led to inhibition of PP2A activity and the resulting DEX-induced GR nuclear translocation (51). In contrast, suppression of miR-9 increased both PP2A activity and GR nuclear translocation in macrophages and restored steroid sensitivity in multiple models of steroid-resistant AHR (51). Furthermore, it was found that miR-9 expression was increased in sputum of patients with neutrophilic but not those with eosinophilic asthma (51). These data underline the critical role of miRNA controlled macrophage activation in the onset of asthma.

As discussed above, the distinct phenotype of Th cells is not only intrinsically modulated, but also controlled by engagement with antigen presenting cells, such as DCs, a process that is subject to regulation by miRNAs. This was first indicated by a recent finding that miR-155/- mice showed reduced eosinophilic airway inflammation compared to wild type counterparts in asthmatic models (121). Further study found that miR-155 deficient DCs demonstrated impaired Th2 priming capacity and failed to induce airway inflammation in allergen exposed wild type mice (121). miR-155 deficiency on DCs was also associated with impaired purinergic receptor signaling, as miR-155 deficient DCs showed reduced chemotaxis and IL-1β secretion upon stimulation with ATP, probably due to direct targeting of ectonucleoside triphosphate diphosphohydrolases (ENTPD) by miR-155 (121). miR-155 has also been shown to inhibit IL-13 induced M2 macrophage polarization by directly targeting IL13Rα1 (61). Given the critical role of M2 macrophages in allergic inflammation, these findings indicate that miR-155 can also inhibit Th2 response by indirectly impacting on macrophage mediated Th2 cell differentiation.
Not only do miRNAs directly participate in Th2 differentiation, they can also serve as mediators for other pro-Th2 activators. It was recently shown that vascular endothelial growth factor (VEGF), a critical regulator of pulmonary Th2 inflammation, downregulates miR-1 expression in the lung, most prominently in the endothelium (101). This study identified Mpl as a direct target of miR-1. In accordance with this finding, VEGF controls the expression of endothelial Mpl during Th2 inflammation, which is dependent on miR-1. More importantly, in vivo knockdown of Mpl inhibited Th2 inflammation (101). These data suggest that not only Th cells themselves, but also other cell populations, including pulmonary endothelial cells, are critical players in Th2 inflammation. All of these cellular events have been described to be subject to miRNA regulation.

Although most efforts have been focusing on the role of miRNAs in CD4+ T cells, there is a recent study showing that the regulation of CD8+ T cell activation by miRNAs also serves an important role in severe asthma. Lindsay et al. found surprisingly that severe asthma is associated with the activation of circulating CD8+ but not CD4+ T cells (104). This response was correlated with the downregulation of miR-146a/b and miR-28-5p. They also discovered expression alteration of multiple lncRNA species in the CD8+ T cells of patients. These preliminary data suggest that CD8+ T cell function can be regulated by ncRNAs, including miRNAs and lncRNAs. However, it remains to be determined how these ncRNAs modulate CD8+ cell function and whether these lncRNAs participate in asthma pathology.

ncRNAs and inflammation in COPD

COPD is characterized by abnormal immune responses of the lung to noxious particles and gases. Exposures to smoke from cigarettes and biomass fuels have been shown to be the primary risk factors for COPD (12). Deleterious substances from these exposures trigger innate immune response through pattern recognition receptors on the surface of macrophages and other immune cells. Activated DCs then stimulate adaptive immune responses encompassing Th (Th1 and Th17) cells, CD8+ cytotoxicity, and B cell responses that also lead to the development of lymphoid follicles on chronic inflammation (12).
Alveolar macrophages are critical components of the innate immune network in the lung and serve key roles in perpetrating inflammation in COPD lungs (27). To study if miRNAs are involved in the effect of cigarette smoke on alveolar macrophage phenotype, two elegant studies from the same group of researchers specifically profiled miRNA expression in alveolar macrophages from smokers and non-smokers (32, 33). They found that many miRNAs demonstrated altered expression in alveolar macrophages of the smokers and there was an overall decrease in global miRNA abundance (32, 33). Additionally, the degree of the global decrease in miRNA expression was associated with smoking history. In accordance, many of the miRNAs with reduced expression in alveolar macrophages of smokers were predicted to target mRNAs upregulated in alveolar macrophages of these subjects (32). Further evidence showed that the decrease in global miRNA abundance was due to a defect in miRNA maturation caused by cigarette smoke (33). They demonstrated that cigarette smoke induced Dicer SUMOylation, a post-translational modification that is mediated by the SUMOylating ligase, Ubc9 (33). These data suggest that miRNAs are required for the homeostasis of macrophage activity in COPD alveolar macrophages.

Professional immune cells are the direct contributors to the persistent inflammation in COPD lungs. However, interstitial fibroblasts are also an integral part of such a non-receding pathology with this disease. COPD fibroblasts have been shown to produce excessive amount of the pro-inflammatory mediator prostaglandin E2 (PGE2) (103). The overproduction of PGE2 was recently demonstrated to be caused by deregulation of miR-146a in COPD lung fibroblasts (89). In this study, Sato et al. found that PGE2 production after treatment with inflammatory cytokines was in correlation to the expression of COX-2, which was a target of miR-146a. miR-146a was also induced by inflammatory cytokines, establishing a negative feedback role this miRNA conducts in controlling PGE2 production in normal lung fibroblasts. However, the regulatory mechanism of COX2 expression involving miR-146a was compromised in COPD fibroblasts as the induction of this miRNA after exposure to inflammatory cytokines was not as robust as that in the normal subjects. These data suggest that restoring miR-146a expression in COPD lung fibroblasts could become a novel therapeutic approach. The first priority in achieving this goal, however, is to unravel the suppressive
mechanism of miR-146 expression in COPD fibroblasts. Indeed, there has already been
evidence indicating the involvement of epigenetic alterations associated with cigarettes smoke.

Alpha-1-antitrypsin (AAT) deficiency constitutes a major cause to emphysema/COPD (113).
AAT mutations decrease serum levels of AAT, which leads to disrupted protease homeostasis
in the lung. This is a well accepted pathogenic mechanism for COPD or emphysema (113).
However, it has been also shown that accumulation of abnormal AAT triggers unfolded protein
response (UPR) in AAT-deficient monocytes, an event that can cause inflammatory activation.
To investigate if miRNAs participate in the mutant AAT induced UPR in monocytes, the group
led by Greene performed miRNA profiling on peripheral blood monocytes isolated from
asymptomatic and symptomatic MM and ZZ subjects (35). They found that 43 miRNAs were
differentially expressed, with miR-199a-5p being the most upregulated in asymptomatic ZZ
versus MM monocytes (35). They further showed that miR-199a-2 promoter hyper-
methylation was increased in symptomatic MM and ZZ monocytes compared with that in
asymptomatic counterparts (35). This might explain the reduced expression of this miRNA in
monocytes from symptomatic individuals. UPR and inflammation mediators, such as activating
transcription factor 6 (ATF6), p50, and p65 were confirmed to be direct targets of miR-199a-
5p. In accordance with this finding, GRP78, ATF6, p50, and p65 were increased in
symptomatic versus asymptomatic ZZ monocytes. miR-199a-5p might also be involved in
regulation of Treg function in COPD patients, as recently indicated by the study from Chatila
et al. (17). They found that miR-199a-5p was repressed by 4-fold in Tregs from COPD patients
compared to those from healthy smokers and this miRNA seemed to participate in TGF-β
activation pathway. However, it is currently unknown how miR-199a-5p regulates Treg
activity, particularly with respect to Treg modulation of Th1 and Th17 activation in COPD.
Regardless, these studies present a typical example that dysregulation of the same miRNA can
occur in multiple types of immune cells to collectively cause relentless inflammation in COPD
lungs.

ncRNAs and immune regulation in CF
Cystic fibrosis (CF) is a genetic disorder characterized by a massive pro-inflammatory phenotype in the lung (41). These include pulmonary infiltration of large numbers of neutrophils and high levels of pro-inflammatory cytokines in the lung, such as IL-8 (41). Bronchial epithelial cells constitute the primary contributors to the pulmonary inflammation manifested in CF (21). Although many regulatory mechanisms are involved in the pro-inflammatory phenotype of CF epithelial cells, evidence is growing that ncRNAs are crucial nodes located in the network of immune signaling in these cells (9). An early report of one such example showed that miR-126 expression was reduced in CF versus non-CF airway epithelial cells both in vitro and in vivo (81). miR-126 directly targeted TOM1 (target of Myb1) in bronchial epithelial cells, a protein that has been shown to interact with Toll-interacting protein and likely function as a negative regulator of inflammatory signaling. Consistently, overexpression of TOM1 downregulated NF-κB luciferase activity in LPS treated airway epithelial cells, whereas knockdown of TOM1 significantly increased NF-κB dependent IL-8 secretion (81). These data suggest that miR-126 downregulation in CF airway epithelial cells is a protective mechanism to curb excessive inflammation.

A later improved study systemically examined the expression profile of miRNAs in lung epithelial cell lines that carry ΔF508-CFTR or wild type CFTR and found that the expression of miR-155 was increased more than 5-fold in CF IB3-1 lung epithelial cells compared to that in control IB3-1/S9 cells (8). They showed that miR-155 promoted IL-8 production by targeting the SHIP1-Akt axis in lung epithelial cells (8). Along with findings in numerous other inflammatory settings, these data firmly establish miR-155 as a master regulator of immune response in the lung, which indicates many opportunities of therapeutically targeting this miRNA.

There were two miRNAs that were demonstrated to directly target IL-8 in airway epithelial cells (29, 82). Oglesby et al. showed that expression of miR-17 was decreased in adult CF bronchial brushings, β-ENaC transgenic mice, and bronchial epithelial cells treated with Pseudomonas-conditioned medium. Overexpression of miR-17 inhibited basal and agonist-induced IL-8 production in F508del-CFTR homozygous CFTE29o° tracheal, CFBE41o° and/or IB3 bronchial epithelial cells (82). In another study, Fabbri et al. found that
miR-93 expression was decreased in *Pseudomonas aeruginosa* infected CF bronchial epithelial IB3-1 cells (29). They showed that overexpression of miR-93 blunted *Pseudomonas aeruginosa* infection induced IL-8 production, whereas blocking miR-93 upregulated IL-8 in uninfected airway epithelial cells (29). Combined, these findings identified two miRNAs as potential therapeutic targets for controlling refractory inflammation in CF lungs.

miRNAs also regulate the antimicrobial activity in the lungs of CF patients. Weldon and colleagues discovered that miR-31 levels were decreased in the CF airways (112). miR-31 targeted IRF-1 in CF epithelial cells, a transcriptional factor that activates the expression of Cathepsin S (CTSS) (112). They further showed that CF airway epithelial cells expressed and secreted significantly more CTSS than non-CF control cells in the absence of pro-inflammatory stimulation (112). In accordance, miR-31 mimics decreased IRF-1 protein levels with concomitant reduction in CTSS expression and secretion in CF bronchial epithelial cells (112). Given the activity of CTSS in degradation of antimicrobial proteins, such as lactoferrin and members of the β-defensin family, targeting miR-31 could represent a novel therapeutic strategy to boost immunity to repeated infections in CF lungs, a condition recognized as a primary culprit to cause declined lung function in CF patients.

Reduced levels of the scaffold protein caveolin 1 (CAV1), a negative regulator of TLR4 signaling, in macrophages have been shown to cause hyper-inflammatory response to LPS in CF mouse lungs. A recent study implicated miR-199a-5p in the causes to CAV1 downregulation (123). It was shown that miR-199a-5p was upregulated in CF macrophages and miR-199a-5p knockdown rescued CAV1 expression and diminished hyper-inflammation in these cells (123). Of the most significant findings in this study, the FDA-approved drug celecoxib reversed the pattern of miR-199a-5p and CAV1 expression in CF macrophages and diminished lung hyper-inflammation in *Cftr*-deficient mice (123). These data suggest that overly activated innate immune response caused by dysregulated miRNAs plays a major role in the occurrence of uncontrolled inflammation in CF lungs.

Although we have gained a larger insight into the contribution of miRNA regulated immunity to CF pathogenesis, the role of lncRNAs in the inflammation of CF lungs is only beginning to
unravel. Greene's group examined the expression of 30,586 lncRNAs by microarray in bronchial cells and identified a total of 1,063 lncRNAs with differential expression between from CF and non-CF individuals (67). Bioinformatics analysis revealed that many pathways enriched in the CF bronchial epithelium are related to inflammation. Their data suggest that dysregulation of lncRNAs may serve essential roles in the chronic infection and inflammation in CF lungs.

**ncRNA regulation of immune response in ARDS/ALI**

There were several recent studies that all focused on the impact of miRNA regulated macrophage polarization on various types of acute lung injuries. Ying et al. reported a pivotal role of miR-127 in macrophage differentiation and thereby the pathogenesis of inflammation and lung injury (120). They discovered that miR-127 expression was upregulated by TLR ligands and downregulated by Th2 cytokines (120). Overexpression of miR-127 in macrophages increased the production of pro-inflammatory cytokines, whereas inhibition of miR-127 led to decreased M1 gene expression and a M2 leaning phenotype (120). More significantly, miR-127 mimics promoted LPS induced pulmonary inflammation and injury in mice. In contrast, blocking miR-127 protected mice from LPS induced ALI (120). In delineation of the pro-inflammatory mechanism of miR-127, they showed that miR-127 targeted B cell lymphoma 6 (Bcl6) and Bcl6 dependent expression of dual specificity phosphatase 1 (Dusp1), a phosphatase which is inhibitory to the pro-inflammatory kinase JNK (120). In accordance, reconstitution with the expression of Bcl6 or Dusp1 or inhibition of JNK reversed miR-127 enhanced M1 macrophage differentiation, whereas knockdown of Bcl6 or Dusp1 expression abolished the anti-inflammatory activity of anti-miR-127 (120). Their findings highlight the critical role of macrophage polarization in lung inflammation and injury and a master control of these events by a single miRNA. Although the pro-inflammatory role of miR-127 in LPS induced ALI seems well established, another study showed that miR-127 attenuated lung inflammation induced by IgG immune complex via targeting IgG FcγRI (CD64) (119). These opposing effects of miR-127 found in the two models could be explained by a scenario where miR-127 may have distinct roles in different lung cell populations. miRNA regulated macrophage activation has also been proven to contribute to the actions of
other pro-inflammatory kinases in ALI, such as Akt2. Vergadi et al. found that alveolar macrophages demonstrated M1 phenotype in WT mice, but M2 phenotype in Akt2-/- mice, after acid-inflicted lung injury (108). Correspondingly, acid-induced lung injury was less severe in Akt2-/- mice compared with WT mice (108). They further showed that miR-146a prevented M1 activation \textit{in vitro} and \textit{in vivo} and is critical for the protection from acid induced ALI found in mice with Akt2 deletion (108).

In another study, Guo et al. showed that miRNA regulated macrophage activation can also regulate lung inflammation through affecting other cell populations (34). Their study demonstrated a protective effect of antisense oligonucleotides (ASOs) against miR-155 on LPS induced ALI in mice, which was conferred by increased numbers of CD4^+CD25^+ Tregs in these mice (34). Further evidence showed that the amplification of Tregs in the miR-155 ASO-treated ALI mice was dependent on IL-10 secreting M2 macrophages in these animals (34).

Consistent with previous studies, they showed that miR-155 targeted C/EBP-β, a pro-M2 transcriptional factor (34, 54). As expected, C/EBP-β expression was upregulated in the miR-155 ASO-treated ALI mice (34). These data indicate that there exists a network of inflammatory cells in the lung that impact the functional phenotype of each other through miRNAs in the duration of acute inflammation.

Not only are classical inflammatory cells the key players in the pathogenesis of lung inflammation and injury, activation and dysfunction of endothelial cells (ECs) are also essential prerequisites for this disorder (96). A study from Feinberg et al. underlined the importance of miRNAs in EC activation and inflammation as well as lung injury associated with these events (97). They found that miR-181b was a potent negative regulator of NF-κB signaling in the vascular endothelium by targeting importin-α3, a protein that mediates nuclear translocation of NF-κB (97). Overexpression of miR-181b inhibited importin-α3 expression and downregulated NF-κB-dependent expression of pro-inflammatory mediators, such as adhesion molecules VCAM-1 and E-selectin, in ECs in \textit{vitro} and in mouse lungs (97). More importantly, supplement of miR-181b in the lung by systemic delivery of miR-181b mimics blunted NF-κB signaling and ameliorated lung injury and mortality in mice with LPS induced
endotoxemia. In contrast, blocking miR-181b aggravated these processes (97). These data supported the findings that critically ill subjects with sepsis had decreased levels of miR-181b.

595  ncRNA based therapeutics in inflammatory lung diseases

596  Compared to IncRNAs, miRNAs are easy targets for therapeutics due to their tiny size and relatively conserved sequences (105). Although targeting miRNA in animal models has been used to characterize the role of individual miRNAs in several cases of inflammatory lung disorders as described above, the development of miRNA based therapeutics in these lung conditions has lagged behind that in other areas of inflammatory/infectious diseases. For example, there are already several clinical trials underway to test miR-122 antisense oilgos in inhibiting HCV replication in the liver (38) (http://www.regulsrx.com).

604  The goal of miRNA therapeutics is to restore normal expression of dysregulated miRNAs. There are currently two approaches to reconstitute downregulated miRNAs in vivo. One is to use synthetic modified double-stranded miRNA mimics and the other is to use lenti-, adeno, or adeno-associated virus to produce individual miRNA (105). The most employed and efficient approach to block upregulated miRNAs is to use synthetic single-stranded antisense oligos called antimiRs (105). However, these antimiRs need carefully designed chemical modifications to achieve high binding affinity, biostability and pharmacokinetic properties (105).

614  Although numerous ncRNAs, particularly miRNAs, have been found to partake in the aberrant immune response associated with many lung diseases, it remains a great challenge to therapeutically target these ncRNAs in a specific type of immune cells. Additionally, since miRNAs generally regulate multiple targets and are known fine-tuners of cellular and molecular processes, it is critical for therapeutic interventions to restore their physiological levels to avoid unwanted side effects. Altogether, given the excellent efficacy that miR-122 antisense oilgos have demonstrated in treating HCV infections (38), there is an optimism that a new era of miRNA based therapeutics in lung diseases is coming in the not too distant future.
Conclusion and perspectives

The past decade has witnessed the great expansion in the diversity of gene regulation. With the role of ncRNAs in immune system becoming clearer, there is a certainty that the acquired knowledge will translate into the understanding how this type of molecules affect the initiation, progression and resolution of inflammatory lung diseases. However, ncRNAs have been generally regarded as another layer of gene regulation and the delineation of their roles literally relies on the understanding how they regulate protein targets. This stereotypic approach will continue unless fundamentally new functional mechanisms emerge.

Although there are numerous miRNAs that have been identified to participate in almost every aspects of the innate and adaptive immunity and a wealth of knowledge established in understanding miRNA regulation of these processes, the role of many these miRNAs in inflammatory lung disorders is much less appreciated. Therefore, answering this type of questions should and will remain the focus of future studies in the field. With the delineated regulating processes and identified targets for these miRNAs as well as the availability of many genetically modified mouse strains, we are surely in better position to address these matters in the context of inflammatory lung pathologies. However, there are certainly miRNAs that are uniquely involved in these lung conditions. Identification of these miRNAs will rely heavily on cell type specific profiling analysis. With regard to IncRNAs, there will be a lot more obstacles in defining their roles in inflammatory lung diseases, not only because none of IncRNAs has been characterized in these lung conditions, but also the understanding of their functions in immune response is still in its infant stage. Nevertheless, further examination of ncRNAs' contributions to abnormal immunity in the lung will have profound impact on future clinical interventions due to opportunities to identify numerous novel therapeutic targets.

It is sure that more ncRNAs will be discovered to regulate each step in the immune response to pathogens. However, more pressing questions in future studies include how miRNAs, IncRNAs, and proteins orchestrate their actions in individual immune cells during the processes and how the ncRNA regulated events collaborate in different elements of the
immune network. Clarification of these questions will no doubt advance our understanding why dysregulation of ncRNAs leads to immune disorders in the lung.
Acknowledgements

a) Acknowledgments: we apologize to colleagues whose work could not be cited due to space limitations; we thank Danni Zhou for editorial assistance. b) Sources of funding: NIH grants HL114470, HL105473 and HL076206. c) Financial disclosure: none.
References


Figure legend

**Figure 1**: ncRNAs play pivotal roles in the differentiation, activation, and function of immunological cells. Dysregulation of specific ncRNAs leads to unsynchronized immune response that can cause tissue immunopathologies, such as several lung diseases that are characteristic of aberrant immune response.