New mechanisms in diisocyanate-mediated allergy/toxicity: are microRNAs in play?

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Purpose of review
To describe recent findings of diisocyanate-mediated mechanisms in allergy and toxicology by addressing the role of microRNA (miR) in immune responses that may contribute to the development of occupational asthma (OA).

Recent findings
Studies of diisocyanate asthma have traditionally focused on the immune and inflammatory patterns associated with diisocyanate exposures; however, recognized knowledge gaps exist regarding the detailed molecular mechanism(s) of pathogenesis. Recent studies demonstrate the critical role endogenous microRNAs play as gene regulators in maintaining homeostasis of the human body, and in the pathophysiology of many diseases including asthma. Given that diisocyanate-OA shares many pathophysiological characteristics with asthma, it is likely that miR-mediated mechanisms are involved in the pathophysiology of diisocyanate-OA. Recent reports have shown that changes in expression of endogenous miRs are associated with exposure to the occupationally relevant diisocyanates, toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI). Continued mechanistic study of these relevant miRs may lead to the development of novel biomarkers of occupational exposure and/or provide efficacious targets for therapeutic strategies in diisocyanate asthma.

Summary
The molecular mechanisms underlying diisocyanate-OA pathophysiology are heterogeneous and complicated. In this review, we highlight recent research into the roles and potential regulation of miRs in diisocyanate-OA.

Keywords
4,4′-methylene diphenyl diisocyanate, diisocyanates, microRNA, occupational asthma, toluene diisocyanate

INTRODUCTION

Diisocyanates, including toluene diisocyanate (TDI), methylene diphenyl diisocyanate (MDI), hexamethylene diisocyanate (HDI), and isophorone diisocyanate (IPDI), are a group of highly reactive, low molecular weight chemicals widely utilized as cross-linkers in polyurethane production for diverse commercial and consumer applications. Due to both their ubiquity and strong sensitizing potential, diisocyanates have long been considered the leading cause of chemically induced occupational asthma (OA). The two most widely used diisocyanates, TDI and MDI (Fig. 1), account for more than 90% of the total global market [1]. Exposure to TDI and MDI occurs not only in occupational settings [2,3], but also via incompletely cured consumer products such as memory foam bedding and spray foam sealants [2,3]. The first cases of diisocyanate-associated OA were identified in the early 1950s soon after the introduction of diisocyanates in industry [4], and the number of diagnosed patients continued to increase with an estimate prevalence rate of 5–15% among exposed workers until the 1990s [5–7]. Due to increased use of protective measures among workers since the early 2000s, the incidence rate of diisocyanate-OA has decreased; however, this decrease has plateaued and the number of diagnosed

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KEY POINTS

- MicroRNA (miR)-mediated gene regulation may contribute to diisocyanate allergy and toxicity.
- *miR-155-3p* has been shown to contribute to airway inflammation in toluene diisocyanate (TDI)-occupational asthma (OA); however, the mechanism is not fully understood.
- *miRs-206-3p* and *-381-3p* are downregulated in macrophages exposed to methylene diphenyl diisocyanate (MDI) and MDI-conjugated glutathione.
- *PPP3CA*/Calcineurin A is an endogenous target of *miRs-206-3p* and *-381-3p*, resulting in activation of the nuclear factor of activated T cells (NFAT)-mediated signaling pathway.
- Krüppel-like factor 4 (*KLF4*) is an endogenous target of *miRs-206-3p* and *-381-3p*, which activate the KLF4-mediated signaling pathway.

cases of diisocyanate-OA has remained steady in recent years [8]. Despite the demonstrated public health risk associated with diisocyanate exposure, the underlying pathophysiological mechanisms responsible for diisocyanate-OA remain unclear.

The clinical presentation of diisocyanate-OA, such as the latency period followed by acute and delayed reaction following exposure, is suggestive of an immune-mediated disease [9–11]. Unreacted diisocyanates are not themselves immunogenic, therefore it has long been assumed that xenobiotic diisocyanates react with endogenous biomolecules to form haptenated conjugates which are subsequently recognized by the immune system and elicit an immune response. Although *in vivo* and *in vitro* approaches have identified a variety of diisocyanate-conjugated biomolecules including protein(s) and glutathione (GSH) following exposure, the precise molecular mechanism(s) by which these diisocyanate-conjugated self-molecules initiate signaling transduction cascades, mediate immunological gene expression and ultimately lead to development of OA is still unclear and remains an active research area.

In recent years, microRNAs (miRs), have received significant attention for their demonstrated importance in the pathophysiology of several diseases via posttranscriptional regulation of genes as well as for their potential role as biomarkers in disease diagnosis [12]. Today, miR-relevant research is emerging as a major research area in immune-related diseases. Dysregulation of miRs may contribute to the pathogenesis of various lung and allergic diseases including asthma [13]; however, limited studies are available in the literature on miR-mediated diisocyanate-allergic

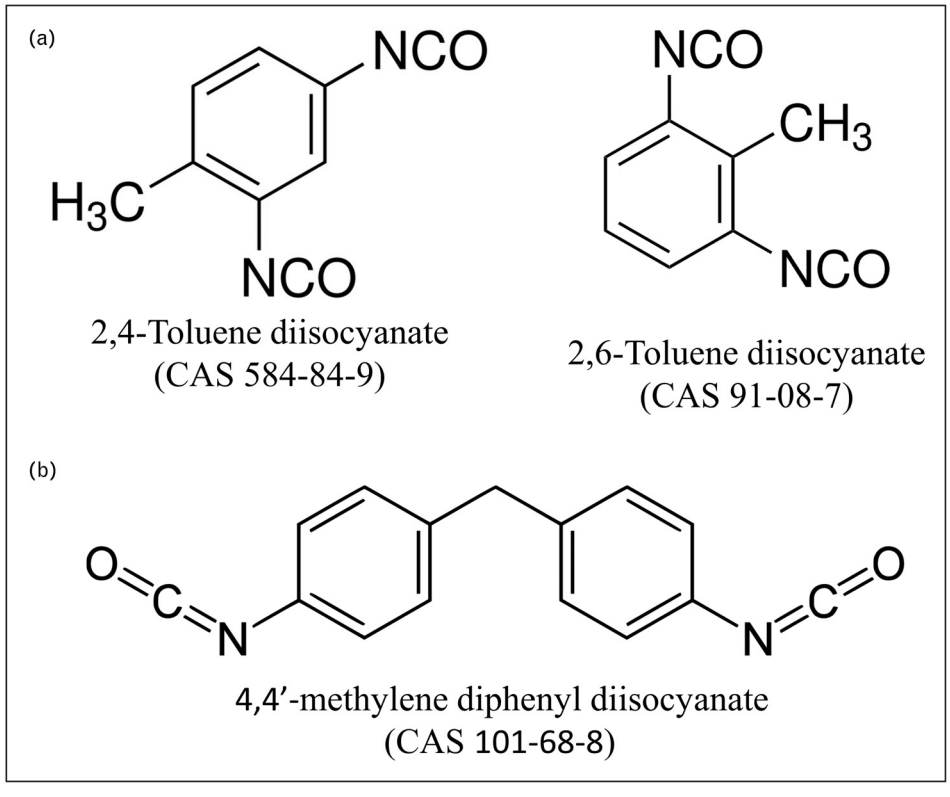

FIGURE 1. Chemical structure of (a) toluene diisocyanate (TDI) including 2,4-TDI, and 2,6-TDI and (b) 4,4'-methylene diphenyl diisocyanate (MDI).

diseases. This review will highlight recent advances toward understanding the role of miRs in diisocyanate-mediated OA with a special focus on exposure to the industrially relevant diisocyanates TDI and MDI. We summarize recent reports of changes in miR expression following TDI/MDI exposure and discuss the potential immunological regulatory mechanisms altered by miRs that may contribute to the pathogenesis of OA. Finally, we explore potential endogenous regulators that could serve as intermediate effector/regulator responses to diisocyanate exposure that may alter miR expression after exposure.

MICRORNA: AN IMPORTANT EPIGENETIC REGULATOR FOR DIISOCYANATE-ASSOCIATED OCCUPATIONAL ASTHMA?

MicroRNAs are single-stranded, noncoding RNAs that typically range from 19 to 25 nucleotides in length and result from extensive processing of the endogenous hairpin-shaped structure of the RNA transcript [14]. Following their discovery in the 1990s, miRs were identified as important regulators of gene expression and implicated as key regulators in a wide range of cell processes including allergic diseases and asthma [15–17]. In mammalian cells, miRs act as master posttranscriptional regulators that control many cellular functions. MicroRNAs usually function as a guide molecule in posttranscriptional gene silencing through partial/imperfect complementary binding to a miR responding element (MRE) located in the 3′ untranslated region (UTR) of the target mRNA, leading to translational repression or RNA degradation. Genes encoding miRs are usually transcribed in the nucleus by RNA polymerase II into primary transcripts of various length, called primary microRNAs (pri-miRs). Multiple proteins are involved in miR maturation, including DROSHA, DGCR8, RAN GTPase/Exportin-5, TARBP2, PACT, and DICER as well as some transcription factors carrying miR maturation regulatory roles includ-

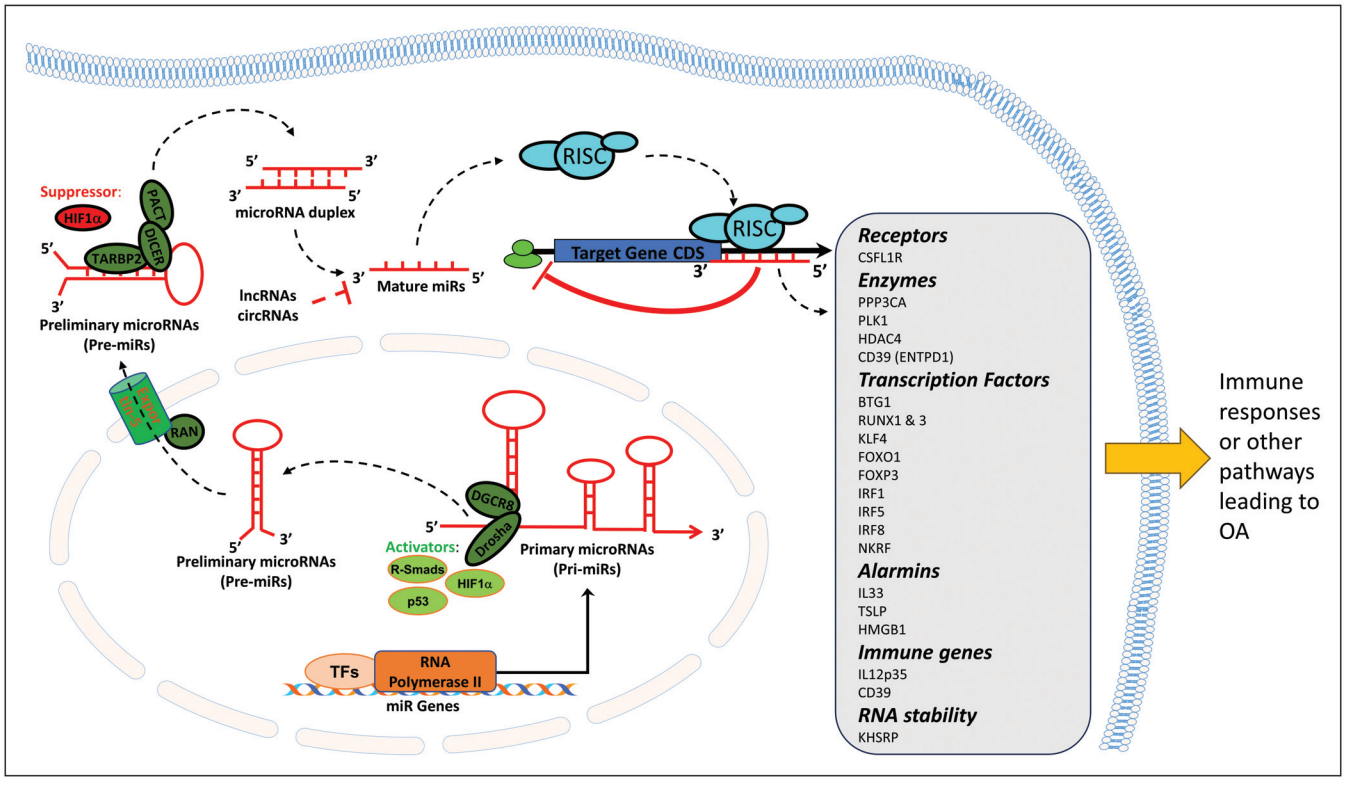

FIGURE 2. Schematic of microRNA biosynthesis, processing, and maturation as well as potential downstream targets regulated by diisocyanate-associated miRs (see Table 1, Supplemental Digital Content, http://links.lww.com/COAI/A40.) after exposure to TDI [25] or MDI [28▪,30▪–32▪]. Following cellular exposure to TDI/MDI, exposure-mediated activation/inhibition of signal transduction pathways recruits transcriptional factors (TFs) to promote/inhibit the expression of primary-miR transcripts. Activators/suppressors of miR maturation and noncoding RNA species (lncRNAs and circRNA) of miR degradation may be recruited after TDI/MDI exposure to regulate expression of mature miRs in the cytosol. TDI/MDI exposure-mediated miRs thus target and regulate multiple mRNA transcripts encoding proteins that may play important roles in immune response, transcriptional regulation or other pathways leading to OA. *Note.* Some illustrated schematics were obtained from *motifolio* templates (www.motifolio.com, Accessed July 8, 2024).

ing R-SMADs [18], P53 [19], and HIF1α [20,21] (Fig. 2). Mature miRs are recruited into the RNA-induced silencing complex (RISC), a multiprotein complex containing Argonaute (Ago) endonucleases [14]. Once miR-containing RISCs are formed, miRs guide the RISCs to the MRE of the target transcripts and may regulate gene expression through mRNA degradation, resulting in the downregulation of the target protein. Because of the imperfect binding of the miR to the MRE, multiple RNA transcripts may be targeted/regulated by one particular miR at the same time [14]. Thus, dysregulation of miRs may affect specific cellular responses and contribute to the development of many diseases, including asthma [22]. MiRs have been shown to be critical to the pathogenesis of different endotypes of asthma [23,24]; therefore, it is likely that miR-mediated mechanisms also underlie diisocyanate-OA.

Putative role of microRNA in diisocyanate exposure associated diseases

At the time of this review, relevant studies have focused on changes in miR expression profile in animal models of TDI/MDI exposure or the use of *in vitro* cell culture models to study the molecular mechanisms of specific miRs and their targets following TDI/MDI exposure [25–27,28▪,29,30▪–32▪] (*Note*. Changes of miRs after TDI/MDI-exposure and their potential target genes that may participate in OA pathogenesis are summarized in Table 1, Supplemental Digital Content, http://links.lww.com/COAI/A40 and shown in Fig. 2). In 2014, Anderson *et al*. published the first report identifying changes in miR expression following dermal TDI exposure in a mouse model [25]. Using both microarray and RT-qPCR methods to analyze changes in the miR profile, the authors identified that dermal TDI exposure leads to consistent changes in the expression of murine *miRs-21-5p, -22-3p, -27b, -31-5p, -126-3p, -155-3p, -210-3p*, and *-301a-3p* in the draining lymph nodes of TDI-exposed animals [25]. Using *in silico* analysis of common mRNA targets, RT-qPCR, and ELISA assays, the authors determined that cytokines IL-5, IL-13, and IFNγ, as well as transcription factors *Runx1* and *Foxp3* were associated with identified miR expression kinetics and may attribute to TDI dermal sensitization [25].

Mitchell *et al*. first reported that circulating miRs may be useful as cancer biomarkers [33]. Since then, circulating miRs have been investigated as an important class of novel, minimally invasive biomarkers for a variety of diseases such as asthma [34]. Lin *et al*. published a report suggesting that expression patterns of circulating miRs in the serum may serve as novel biomarkers for MDI exposure in murine models [27]. In that report, the authors identified three circulating miRs, *miRs-183-5p, -206-3p*, and *-381-3p* that may serve as novel biomarkers for MDI exposure [27]. Expression of circulating *miR-183-5p* was increased whereas expression of *miRs-206-3p* and *-381-3p* were decreased in sera isolated from MDI-exposed animals. Although this report suggested the potential use of circulating miRs as biomarkers for MDI exposure, the use of miR-based diagnosis in the clinical setting warrant further studies. Further studies designed to validate the use of circulating miRs as biomarkers for diagnosis of MDI exposure and MDI-OA are required.

miR-155-3p

Following the initial report by Anderson *et al*., *miR-155-3p* became a focus of TDI exposure-related miR research [25,29]. Dermal exposure to TDI increases the expression of *miR-155-3p* in mouse parotid draining lymph nodes [25], suggesting that it may play an important role in TDI-OA. Zhou *et al*. reported *miR-155-3p* was associated with asthma pathogenesis [35], and in support, a recent report demonstrated the importance of *miR-155-3p* using a TDI-OA mouse model [29]. Wildtype mice exposed to TDI demonstrated increased numbers of airway goblet cells, bronchoalveolar lavage (BAL) eosinophils, CD4-positive T-cells, type 2 innate lymphoid cells (ILC2s) and airway hyperresponsiveness; however, the TDI-induced inflammatory response and airway hyperresponsiveness were attenuated in *miR-155-3p* knockout mice [29]. Further, murine *miR-155-3p* has been shown to be a critical regulator of IL-33 signaling [36]. IL-33 is one of the alarmins (along with IL-25, TSLP, and others) released by damaged epithelial cells. Alarmins activate innate lymphoid cells (ILCs) and T-helper (Th) cells in mucosal tissues to produce cytokines that contribute to asthma pathogenesis [37]. A prior report showed that TDI exposure resulted in ILC2 and dendritic cell recruitment into the lung in a murine model of TDI asthma [38]. Several human studies and mouse models of atopic asthma suggest that the ILC2 population plays an important role in asthma pathogenesis [39,40]; however, the specific role of this cell population in diisocyanate-OA requires further investigation. *MiR-155-3p* has been shown to affect ILC2 expansion, survival, and function in allergic responses [36,41,42], but, whether *miR-155-3p* and ILCs contribute to airway responses in response to diisocyanate exposure remains to be determined.

miR-183-5p

In murine models, circulating *miR-183-5p* levels in the serum were upregulated following MDI exposure

[27]. During allergic inflammation, B-cell translocation gene 1 protein (BTG1) mRNA transcript was identified as one of the targets that can be regulated by *miR-183-5p,* where BTG1 inhibited *in vitro* allergic reactions [43]. Elevation of circulating *miR-183-5p* levels after MDI-exposure may potentially induce allergic reactions through inhibition of BTG1 in MDI-OA, however, this potential regulatory axis requires further investigation. Circular RNA *mmu_circ_0001359* downregulates *miR-183-5p* in alveolar macrophages and appears to attenuate airway remodeling by enhancing FoxO1 signaling-mediated M2 macrophage activation [44]. The FoxO1 transcription factor plays an important role in the regulation of alternative macrophage activation [45], and given that M2 macrophage populations are elevated in asthmatic airways and are implicated in asthma pathogenesis [46,47], MDI-mediated downregulation of *miR-183-5p* in macrophages may contribute to diisocyanate-OA pathogenesis through FOXO1-mediated M2 macrophage polarization. Our understanding of the detailed molecular mechanism and signaling pathways that are regulated by *miR-183-5p* is currently lacking, and further research is required to elucidate its function in diisocyanate-OA.

miRs-206-3p and -381-3p

Lin *et al.* identified a pattern of downregulated circulating *miRs-206-3p* and *-381-3p* in the serum of MDI-exposed mice that may serve as a potential biomarker for exposure [27]. Using *in silico* pathway enrichment analysis, multiple immune-relative pathways were identified that may be enriched by *miRs-206-3p* and *-381-3p*, including Fc gamma R-mediated phagocytosis, Calcineurin/NFAT, chemokine signaling, FMLP, and leukocyte transendothelial migration pathways [27]. The serum level of circulating human *miR-206-3p* has been identified as a potential biomarker to predict exacerbation in childhood asthma [48]. In support of this prediction, a recent report showed downregulated endogenous *miR-206-3p* in airway epithelial cells isolated from type-2 asthmatic patients [49]. Research has also shown that decreased *miR-206-3p* expression is associated with type-2 inflammation [49], further supporting the finding that type-2 asthma, a major subtype of asthma, is driven by T helper 2 cell-mediated inflammation [50]. In type-2 asthma, the airway epithelial cells play an important role by initiating the secretion of the cytokines interleukin (IL)-25, IL-33, and TLSP [37,51]. Zhang *et al.* determined that *miR-206-3p* targets the CD39-ATP axis and upregulates IL-25 and TSLP expression [49]. Whether or not MDI-exposure mediated downregulation of endogenous *miR-206-3p* can contribute to the induction of CD39 and production/secretion of IL-25 and TSLP in MDI-OA patient requires further investigation.

Recently, studies investigating the role of *miRs-206-3p* and *-381-3p* in macrophages revealed that exposure to both MDI and MDI-glutathione (GSH) conjugate induce the expression and secretion of several chemokines via *miR-206-3p/-381-3p*-mediated pathways and recruit immune cells to the lung microenvironment via chemotaxis (Fig. 3). Exposure to MDI and MDI-GSH decreases endogenous *miRs-206-3p* and *-381-3p* levels [27,28▪] by an unknown mechanism. MDI exposure may cause signal transduction blockage at different levels or may cause DNA damage or inhibit transcription factor activities that mediate the transcription of *pri-miR-206-3p*, and *pri-miR-381-3p*. The primary RNA transcripts are first processed by DROSHA to the precursor form of miRs, that is, *pre-miR-206-3p* and *pre-miR-381-3p*, which translocate into the cytoplasm, and are then processed by DICER to result in the mature form of *miRs-206-3p*, and *-381-3p*. The RISC protein and associated complex guide the miR-RISC complexes to target sites on either the *PPP3CA* or *KLF4* mRNA transcripts, thereby inhibiting the translation of PPP3CA and KLF4, and thereby decreasing endogenous PPP3CA and KLF4 transcription factor expression [28▪,30▪]. Calcineurin is a calcium (Ca^{2+}) and calmodulin-dependent serine/threonine protein phosphatase which is composed of a calmodulin-binding catalytic subunit, calcineurin A (encoded by *PPP3CA*) and a Ca^{2+}-binding regulatory subunit, calcineurin B (encoded by *PPP3R1 or PPP3R2*). Calcineurin is activated by the process of calcium binding to calmodulin. Activated calcineurin dephosphorylates cytosolic nuclear factor of activated T cells (NFAT) transcription factors. Dephosphorylated NFATs and other transcription factors translocate into the nucleus and increase expression of *NOS2, CCL2, CCL5, CCL11, CXCL8*, and other genes [28▪,32▪], which may contribute to the chemotaxis of immune cells into lung microenvironment after MDI exposure.

Not only are both endogenous KLF4 and M2 macrophage-associated markers and chemokines induced following MDI/MDI-GSH exposure in macrophages, but KLF4 was also identified to play an important role in regulating M2 macrophage-associated marker gene transcription [31▪]. Studies conducted by Lin *et al.* determined that mature *miRs-206-3p and -381-3p* regulate the translation of cytosolic KLF4 protein, which then translocates into the nucleus, binds to the promoter region of target genes, and induces M2 macrophage-associated marker genes including *CD206, TGM2, CCL17, CCL22, CCL24*, and others [30▪,31▪]. The increased

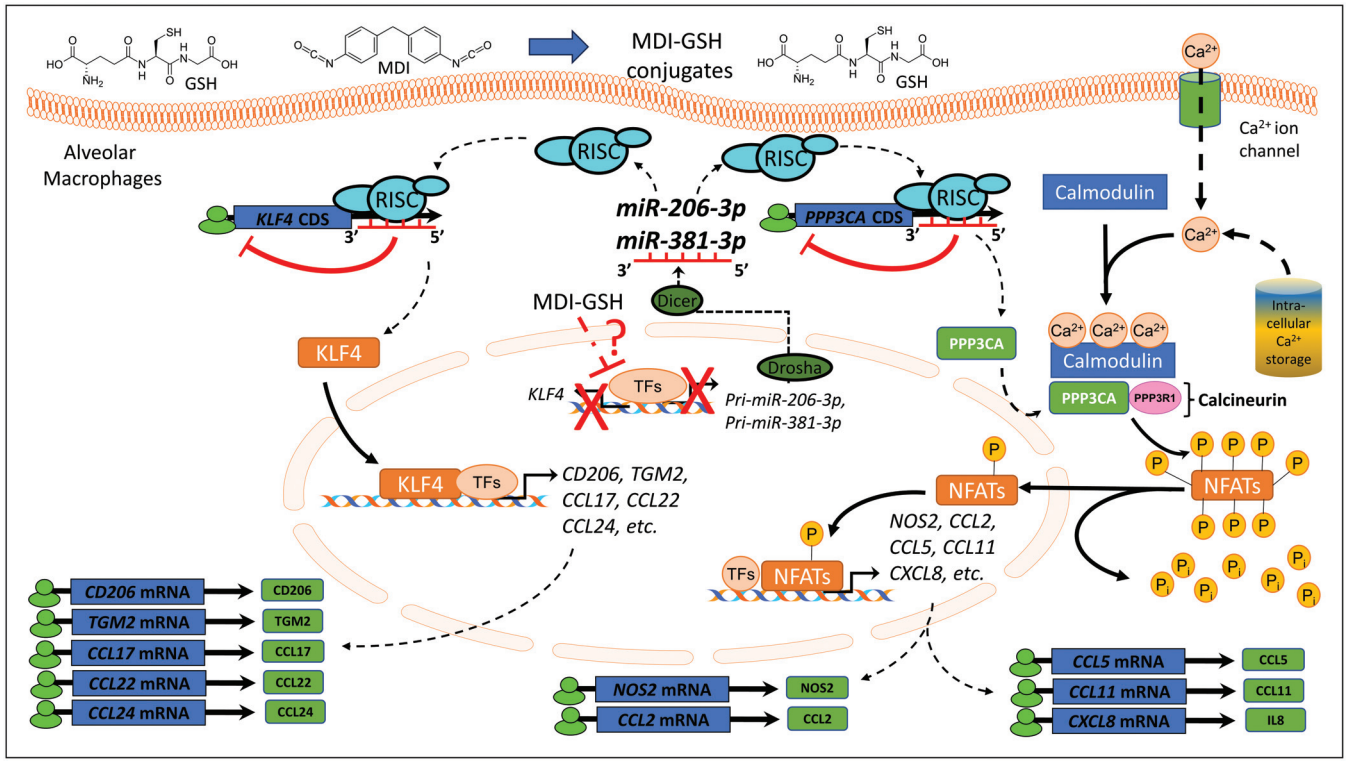

FIGURE 3. Potential miR-mediated marker and chemokine expression following MDI/MDI-GSH exposure in macrophages. *Note.* Some illustrated schematics were obtained from *motifolio* templates (www.motifolio.com., Accessed July 8, 2024).

production of these chemokines induces immune cell chemotaxis in the lung microenvironment, initiating and maintaining lung inflammation. These reports demonstrate that MDI and MDI-GSH exposure decrease endogenous *miR-206-3p* and *-381-3p* levels, elevating endogenous PPP3CA and KLF4, and thereby increasing endogenous iNOS and chemokine expression. This laboratory-observed increase in iNOS expression in response to MDI exposure may explain the clinical observation of elevated fractional exhaled nitric oxide (FeNO) in the airway after MDI exposure. Increased chemokine production may induce immune cell chemotactic ability to the lung microenvironment and initiate inflammation at early stages of asthma and/or maintain inflammation at later stages of asthma pathogenesis.

CONCLUSION AND FUTURE PERSPECTIVES

Over the past three decades, researchers have acquired significant understanding regarding the biological roles and the pathophysiological functions of miRs in both normal physical conditions as well as in disease states. Dysregulation of miR expression may disturb immune responses and participates in the pathogenesis pathways of immune related diseases such as asthma. However, comparatively little is known about miR responses following diisocyanate exposure and their function relative to occupational diseases. This review summarizes the putative roles that miRs may play in the development of diisocyanate-related occupational diseases and discusses the potential immunological associations of miRs with a focus on diisocyanate-OA.

Future diisocyanate-relevant miR research will investigate the mechanisms by which diisocyanate-haptenated biomolecules affect endogenous miR levels and downstream miR-mediated biological processes. Studies designed to evaluate the role of diisocyanate-haptenated biomolecules in the biological processes that regulate miR biogenesis, maturation, and degradation are worthy of investigation, as are the roles of long noncoding RNAs (lncRNA) and circular RNAs (circRNA) in the regulation of endogenous miRs associated with asthma pathogenesis [52]. Deeper understanding of miR function and targets, as well as regulation of endogenous miR maturation and expression, is necessary. Studies investigating miR-associated molecular mechanisms following diisocyanate exposure will provide a better understanding of the molecular mechanisms underpinning diisocyanate-induced OA pathogenesis. Diisocyanate-associated miRs

may prove to be a suitable new class of biomarkers for diagnosing OA or provide new efficacious therapeutic targets for treatment.

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Conflicts of interest

The authors declare that they have no conflicting financial interests. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

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