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Review Article

From mesenchymal stem cells to their extracellular vesicles: Progress and prospects for asthma therapy

Shihan Chen[a,b], *Jianqing Gao*[a,b,c], *Tianyuan Zhang*[a,b,c,*]

[a] *College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China*
[b] *Hangzhou Institute of Innovative Medicine, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China*
[c] *State Key Laboratory of Advanced Drug Delivery and Release Systems, Zhejiang University, Hangzhou 310058, China*

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ABSTRACT

Asthma is a widespread public health concern, with an increasing incidence. Despite the implementation of current treatment strategies, asthma control, particularly for severe cases, remains suboptimal. Recent research has revealed the encouraging prospects of extracellular vesicles (EVs) secreted by mesenchymal stem cells (MSCs) as a viable therapeutic option for alleviating asthma symptoms. Therefore, the present review aims to provide an overview of the current progress and the therapeutic mechanisms of using MSC-derived EVs (MSC-EVs) for asthma treatment. Additionally, different administration approaches for EVs and their impacts on biodistribution and the curative outcomes of EVs are summarized. Notably, the potential benefits of nebulized inhalation of MSC-EVs are addressed. Also, the possibilities and challenges of using MSC-EVs for asthma treatment in clinics are highlighted. Overall, this review is intended to give new insight into the utilization of MSC-EVs as a potential biological drug for asthma treatment.

1. Introduction

Asthma is a kind of chronic inflammatory disease, which shows impacts on both the respiratory tract and lung parenchyma [1]. Asthma is currently presenting a global prevalence of around hundreds of millions of individuals [2]. In particular, 4.2% of adults aged over 20 in China, which means 45.7 million individuals, were reported to suffer asthma [3]. Therefore, asthma has been one of the major public health and healthcare issues. The most recognized pathogenesis of asthma in clinic is airway immune inflammation [4]. The inflammatory cell infiltration, airway hyperresponsiveness (AHR) and airway remodeling caused by immune inflammation after exposure to external stimuli are considered the major reasons for the inducement of asthma [5]. The detailed molecular mechanism of asthma progress has been previously overviewed [6–8]. In clinics, asthma is broadly classified into two categories: type 2 asthma and non-type 2 asthma, which is according to the molecular

* Corresponding author.
E-mail address: tianyuanzhang@zju.edu.cn (T. Zhang).
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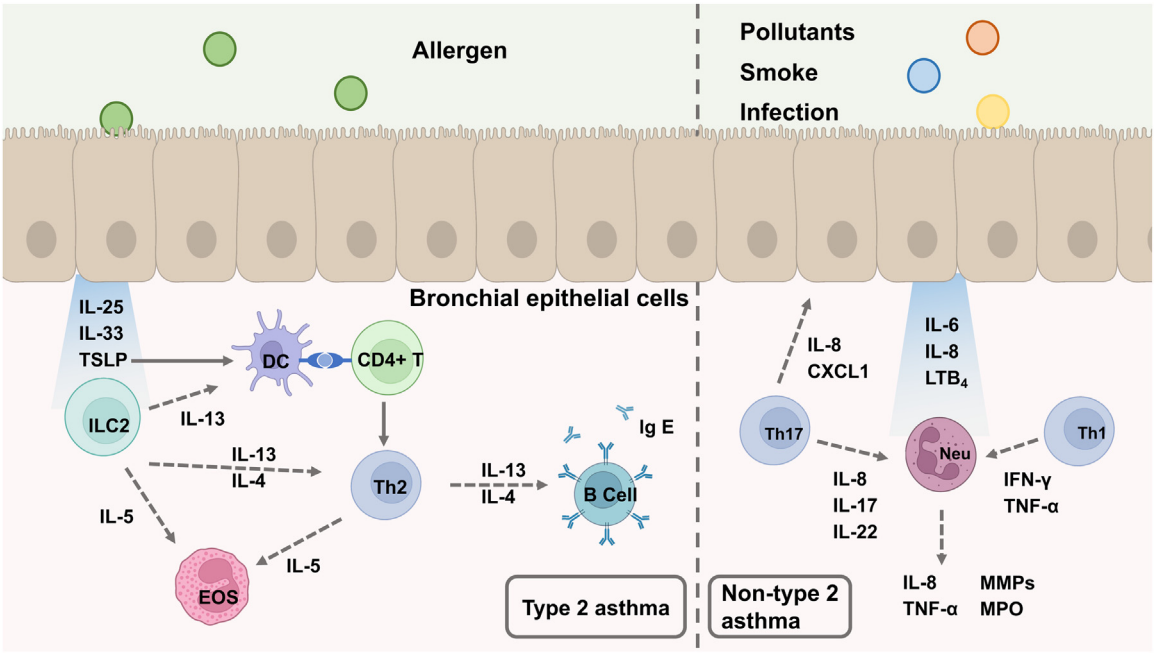

Fig. 1 – Illustration of the pathophysiological mechanism of asthma. Asthma can be classified into two categories according to the molecular mechanism: type 2 asthma and non-type 2 asthma. Stimulations caused by allergen on bronchial epithelial cells could recruit DC and stimulate ILC2s and Th2 to produce type 2 cytokines of IL-4, IL-5 and IL-13. These type 2 cytokines mediate the type 2 inflammation and are responsible for the eosinophil activation and IgE secretion by B cell. As a result, type 2 asthma occurs. By contrast, non-type 2 asthma shows a low level of type 2 inflammation. External stimulations caused by pollutants, cigarette smoke and infection could activate the immune cells, such as Th1 and Th17, to produce non-type 2 inflammatory cytokines (*e.g.*, IFN-γ, IL-6, IL-8, IL-17) and stimulate neutrophilic inflammation in the airways, leading to non-type 2 asthma. (CXCL1: C-X-C motif chemokine ligand 1; LTB4: leukotriene B4; MMP: metallopeptidase; MPO: myeloperoxidase; TSLP: thymic stromal lymphopoietin; EOS: eosinophil; NEU: neutrophil).

mechanism in the asthma progress. Type 2 asthma is a kind of type 2 inflammation and is distinguished by the increase of eosinophilia [9], along with other associated immune cells, including type 2 T helper cell (Th2), B cell and type 2 innate lymphoid cell (ILC2). It has been demonstrated that stimulation caused by an allergen could induce the release of interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin by bronchial epithelial cells [10]. These cytokines not only mediate in the activation of ILC2s but also participate in the phenotypic transformation of dendritic cells (DCs). These DCs can then promote the differentiation of CD4+ *T* cells to Th2 through the allergen presentation [11]. Consequently, the activated ILC2s and Th2 release type 2 cytokines of IL-4, IL-5 and IL-13, which further mediate eosinophil activation through eosinophil-expressed prostaglandin D_2 receptor 2 signaling. The activated eosinophils can release extracellular traps, leading to mucus viscosity and airway inflammation [12]. Moreover, IL-4 and IL-13 also mediate the B cell secretion of immunoglobulin (Ig) E, which further promotes inflammation in the airways. In addition, IL-13 was reported to be associated with enhanced AHR [13]. Different from type 2 asthma, non-type 2 asthma lacks signature biomarkers [14]. Neutrophilic inflammation is currently thought to play a critical role in non-type 2 asthma. Stimulations caused by infection, cigarette smoke and pollution (but not allergens) could activate the immune cells, such as Th1, Th17 and type 3 ILCs, producing diverse inflammation cytokines (*e.g.*, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-6, IL-8, and IL-17), recruiting neutrophils to the lungs, thereby stimulating neutrophilic inflammation in the airways [6]. Furthermore, TNF-α was reported to synergize with IL-17 cytokines, thereby promoting neutrophil recruitment and enhancing airway smooth muscle contraction [15]. Additionally, IFN-γ and TNF-α can upregulate Ca^{2+} signaling in airway smooth muscle and induce AHR, resulting in excessive airway constriction in response to various stimuli. Such pathological process in turn causes significant bronchial stenosis [16]. The major reported mechanisms underlying type 2 and non-type 2 asthma are illustrated in Fig. 1. Currently, type 2 asthma is predominant in clinic and the type 2 inflammation is recognized as the pathophysiological basis of chronic inflammation in asthma [17]. For these reasons, the current review focuses more on the treatment against type 2 asthma.

Bronchodilators and inhaled corticosteroids are presently the major medicants for asthma treatment [18]. However, as non-specific therapeutic medications, they can only suppress asthma symptoms, showing an inability to change the natural course of asthma as well as to reverse the ongoing remodeling process [19]. Moreover, the intolerable adverse reactions, including osteoporosis and hypertension induced by the current medicants, further emphasize the urgent requirements of developing novel therapeutic strategies [20].

Several biologic drugs, such as omalizumab, reslizumab and mepolizumab, have been developed. These drugs can provide patients with individualized treatment at the cellular level and show advantages in reducing the severe asthma exacerbation rate [21]. However, about five to ten percent of asthma patients are still not controlled adequately by the current medications [22]. In addition, most of the current therapies can only suppress the asthma symptoms rather than relieve the asthma through the pathological mechanism, leading to lifelong medication [23]. Therefore, developments of novel therapeutic strategies to recover asthma from pathogenesis are of significance.

Mesenchymal stem cells (MSCs)-based therapy has recently been proposed as a promising strategy for managing asthma mainly by controlling airway inflammation [24,25]. The intrinsic immunomodulatory properties and anti-inflammatory effects of MSCs endow them with potential applications in the treatment of inflammatory diseases [26–28]. Additionally, MSCs are relatively easy to expand *in vitro*, allowing their large-scale production to satisfy the potential clinical applications [29,30]. Nevertheless, MSCs-based therapy is currently facing several challenges due to their inherent shortages. For example, MSCs are inconvenient to store and transport as cellular preparations [31]. Moreover, concerns over the risks of uncontrollable differentiation [28] and short lifespan after administration of MSCs have restricted their further clinical applications [32].

On the other side, several studies have revealed that extracellular vesicles (EVs) produced by MSCs take promise as cell-free therapy and even have the potential as an alternative to MSCs for disease treatment in some conditions [33–35]. This is partly because the therapeutic potentials of MSCs largely rely on their paracrine capability [36]. Several experimental and clinical studies have revealed that the secretome produced by MSCs, including both the soluble components and the encapsulated components in EVs, are principally responsible for the immunomodulatory and anti-inflammatory capabilities of MSCs [37–39]. Therefore, the direct utilization of MSC-derived EVs (MSC-EVs) has been proposed as a promising alternative to MSCs for treatments such as cartilage protection [40] and asthma [28]. However, several challenges, especially the stability, efficiency, and delivery approaches of MSC-EVs are currently hindering the practice of this strategy to treat asthma. Hence, further efforts are required to improve this promising strategy.

The present review aims to provide a thorough summary of the biological effects and therapeutic mechanisms of MSC-EVs for the treatment of asthma, as well as to give an overview of the delivery strategies of EVs and their impacts on therapeutic efficiency. Several previous studies have well summarized the biological properties and the therapeutic applications of stem cell-derived EVs as cell-free therapy [41–43]. Nevertheless, to the best of our knowledge, a detailed overview of using MSC-EVs to treat respiratory or pulmonary diseases, especially asthma treatment, is currently lacking. Also, there are rare reports that have summarized the impacts and efficiency of the administration routes of EVs. Still, this point is particularly important for the disease treatment of the airways and the lungs. Therefore, the insights and findings presented in this review are expected to highlight the novel strategy of utilizing MSC-EVs for asthma treatment and to provide guidance for their future optimization for efficient asthma treatment.

2. MSC-EVs-based therapy for asthma

2.1. *What are EVs and why use MSC-EVs?*

EVs are a generic term for heterogeneous vesicles enclosed by a phospholipid bilayer that are shed into the extracellular space and body fluids by various cell types, such as stem cells, immune cells, epithelial cells, and tumor cells [44]. One of the major roles of EVs is as carriers of biological information. EVs can transport many different biological payloads, including proteins, lipids, nucleic acids, signal transduction molecules, and even organelles such as mitochondria [45]. Through receptor-mediated interactions with target cells [46], EVs have two ways to discharge their interior components: direct endocytosis and membrane fusion [47]. They play crucial roles in regulating normal physiological functions and can have both positive and negative impacts on disease development, being regulators and therapeutic agents in the disease microenvironment [48]. Therefore, significant research interest has been raised in the potential applications of EVs in illness diagnosis and treatment. In addition, continuous efforts have been made to elucidate the critical roles of EVs in intercellular communication and disease pathology [49]. According to the generally accepted classification methods, primary cell-derived EVs can be categorized into three subtypes: exosomes, microvesicles, and apoptotic bodies. Presently, exosomes are the most reported EVs for disease treatment, which are typically in the diameters of 30–200 nm [50]. In the following of the review, EVs are commonly referred to as exosomes.

As previously discussed, despite the promising potential of MSCs for treating asthma, their associated risks for clinical use remain controversial. In addition, several previous studies have shown that the majority of systemic administrated MSCs are eliminated within a few days either by apoptotic or alveolar macrophage-mediated phagocytosis [51]. However, MSCs could exert sufficient therapeutic effects even with a small number of survived cells [52]. This phenomenon indicates that the therapeutic potential of MSCs may not completely rely on the stem cells themselves, and other therapeutic approaches associated with MSCs may also work. Particularly, MSCs were demonstrated to alter the miRNA levels when administered to treat asthma, which was believed to be a potential mechanism in treating asthma [53]. In fact, it has been confirmed that MSCs can regulate immune responses and exert protective effects in asthmatic settings by controlling pro-inflammatory small RNA expression, such as miRNA155, miRNA133, miRNA21 and others [54]. Furthermore, the previous study has indicated that MSCs are capable of transferring small genetic molecules, such as miRNA, through intercellular communication to modulate the phenotype and biological activity of recipient cells [55]. Moreover, EVs have been demonstrated to perform the majority of paracrine functions and are responsible for

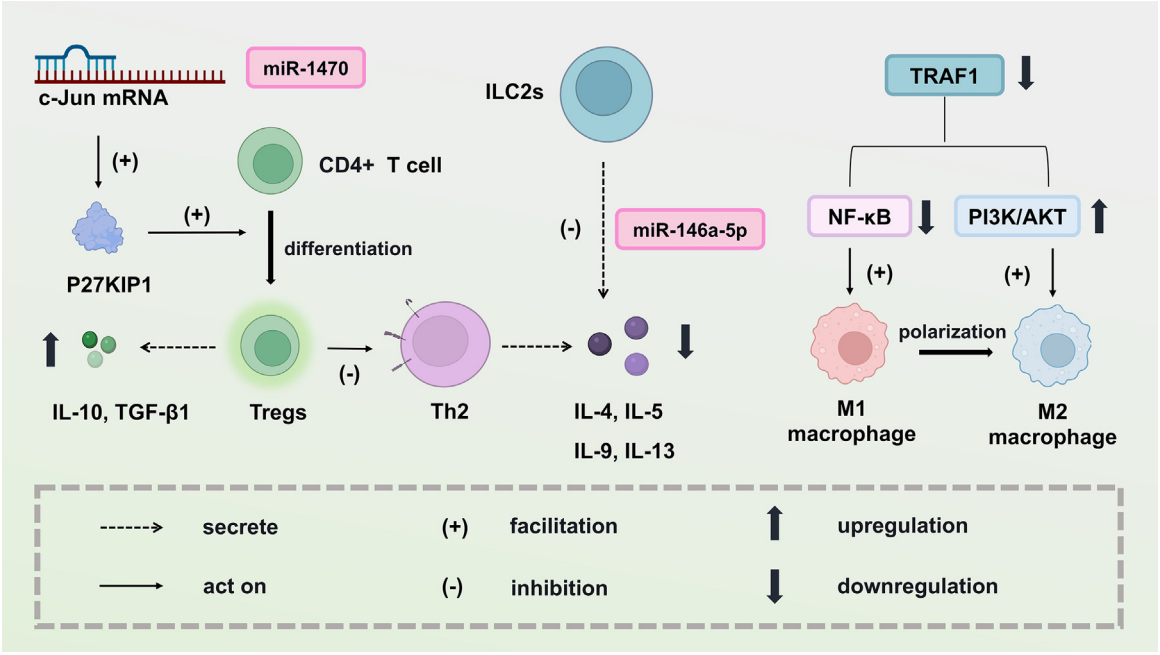

Fig. 2 – Mechanisms of the immune regulation by MSC-EVs in asthma treatment. MSC-EVs are enriched with miRNAs, which can regulate Tregs, ILC2s and macrophages to suppress immune responses. For example, miR-1470 in MSC-EVs can suppress the c-Jun expression by binding to c-Jun mRNA, resulting in the indirect upregulation of P27KIP1 levels. The increased levels of P27KIP1 will promote the differentiation of CD4+ T cells to Tregs, thereby increasing the expression of anti-inflammatory factors (*e.g.*, IL-10 and TGF-β1) and decreasing the expression of inflammatory factors (*e.g.*, IL-4, IL-5, IL-9, and IL-13). Moreover, miR-146a-5p in MSC-EVs can also inhibit the expression of inflammatory factors by ILC2s. In addition, MSC-EVs can downregulate the expression of TRAF1 in macrophages, resulting in the deactivation of NF-κB and the activation of PI3K/AKT, and eventually promoting the macrophage transition from M1 phenotype to M2 phenotype. Consequently, MSC-EVs adjust the inflammatory microenvironment and relieve the symptoms of asthma.

delivering genetic information to recipient cells [56]. All these findings support the view that MSCs can paracrine EVs to exert their therapeutic effects [57,58].

In addition to the therapeutic potential, MSC-EVs show the advantages of avoiding several inherent shortages of using MSCs. First of all, MSC-EVs are widely accepted to have lesser safety risks than MSCs [59]. Moreover, MSC-EVs can be filter sterilized and have a longer shelf life, which is convenient for their production and transportation [60]. Furthermore, the small size of MSC-EVs allows them to avoid the cellular damage caused by shear stress during intravenous injection and prevent the occurrence of pulmonary vascular thrombosis [34]. All these advantages make MSC-EVs a promising candidate for asthma treatment.

2.2. *How MSC-EVs treat asthma?*

MSC-EVs can modulate the biological activity of target cells by either directly activating surface receptors or delivering signaling chemicals into cells [61]. In asthma treatment, the therapeutic mechanisms of MSC-EVs can be categorized into four different major mechanisms: (1) MSC-EVs possess the capability to promote the proliferation of regulatory T cells (Tregs), thereby enhancing the immunosuppression, reducing eosinophils, and suppressing the degree of inflammation in asthma; (2) MSC-EVs can inhibit human ILC2s activity, thus lowering the Th2 cytokines levels, suppressing lung inflammation and lessoning AHR. (3) MSC-EVs contribute to promoting the macrophage phenotype from the pro-inflammatory M1 state to the anti-inflammatory M2 state. (4) MSC-EVs participate in the inhibition of airway remodeling. These four detailed mechanisms are discussed in the following sections and illustrated in Figs. 2 and 3. Irrespective of the involved mechanism, the suppression of lung inflammation and the inhibition of airway remodeling are described as the primary mechanisms of MSC-EVs in asthma treatment. Significant airway inflammation will stimulate bronchial smooth muscle spasms that manifest as chest tightness, wheezing or coughing. Airway remodeling will result in persistent ventilatory dysfunction of the airways, which will eventually lead to dyspnea [62].

2.2.1. *Regulating the level of Tregs*

Tregs are vital participators in maintaining immune tolerance. They can suppress the activation and proliferation of autoreactive T cells, thereby regulating the immune response [63]. One previous research has demonstrated that MSC-EVs contain an abundance of miR-1470 [64]. These miRNAs in EVs were observed to participate in the immunologic pathophysiology of asthma through the regulation of P27KIP1 expression, which can eventually impact the populations of Tregs [65]. Another research has shown that intranasal

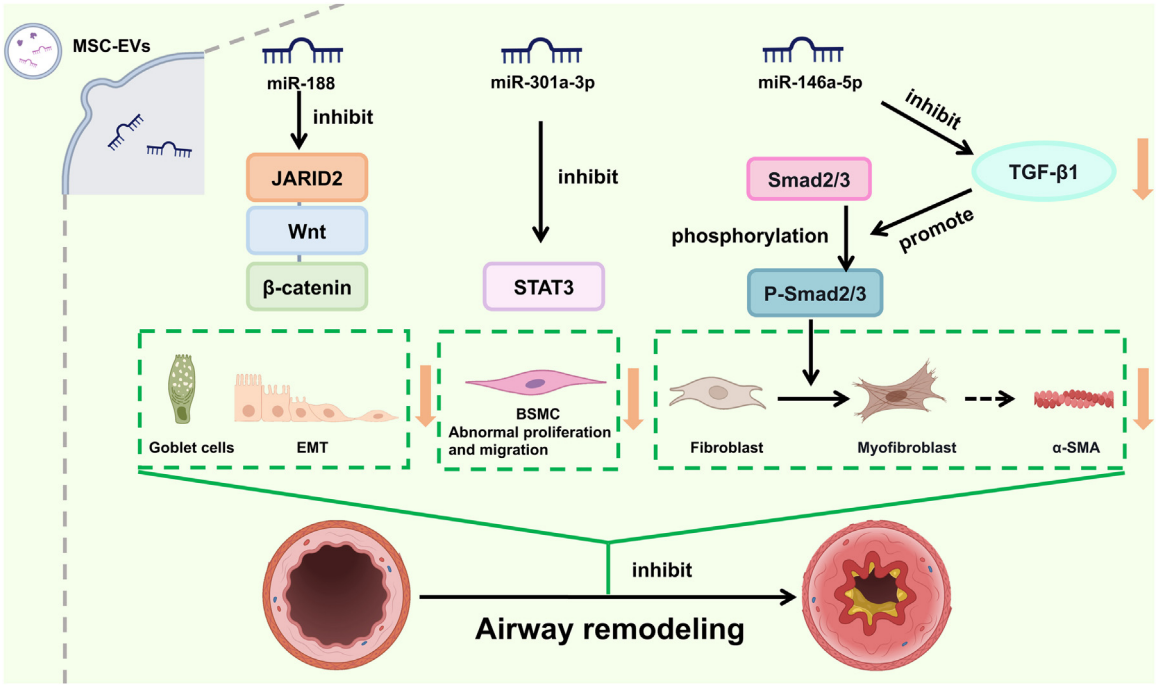

Fig. 3 – Mechanism of MSC-EVs in inhibiting airway remodeling. miRNAs, such as miR-188, miR-301a-3p, and miR-146a-5p, in MSC-EV are responsible for the inhibition of airway remodeling. miR-188 can lead to the reduction of mucin-secreting goblet cells and EMT suppression *via* the inhibition of JARID2/Wnt/β-catenin signaling pathway. miR-301a-3p has demonstrated a potent capability of suppressing the proliferation and migration of BSMCs through the downregulation of STAT3. miR-146a-5p is responsible for the inhibition of TGF-β1, which can further suppress the phosphorylation of Smad2/3, resulting in the mitigation of the transformation of fibroblasts into myofibroblasts and consequently reducing the α-SMA levels. (α-SMA: α-smooth muscle actin; STAT3: signal transducer and activator of transcription 3; EMT: epithelial-mesenchymal transition).

administration of EVs produced by adipose-derived MSCs could reduce the levels of eosinophilic granulocytes and IL-4, IL-13, IL-5, as well as the levels of total IgE and IgG1 in serum. Also, Tregs were detected in increased numbers [66]. Furthermore, Du et al. showed that the EVs produced by human bone marrow-derived MSCs can relieve asthmatic airway inflammation in mice model by modulating immune response through the promotion of Treg proliferation. This function is closely related to the increased expressions of IL-10 and transforming growth factor-β1 (TGF-β1) by MSC-EVs *via* an antigen-presenting cell dependent approach [67].

2.2.2. *Inhibiting the activation of ILC2s*

Previous research has confirmed the critical roles of ILC2s in airway inflammation in type 2 asthma [68]. A piece of typical evidence is that the prevalence of peripheral ILC2s in asthmatic individuals is significantly increased [69]. In addition, the amount of ILC2s was observed to be associated with persistent pulmonary eosinophilia in severe asthma cases [70]. Therefore, inhibiting the activation of ILC2s has been believed to be a potential approach for the treatment of asthma. MSC-EVs were also found to inhibit lung inflammation through this approach. In a previous work performed by Fang et al., MSC-EVs were observed to effectively inhibit the activity of ILC2s, which is partly due to their contained miRNAs, such as miR-146a-5p. The inhibition of ILC2s further suppressed lung inflammation and lessened AHR in mouse ILC2s-dominant asthma model [71].

2.2.3. *Promoting phenotypic transformation of macrophages*

The transition of macrophage phenotype from the pro-inflammatory M1 type to the anti-inflammatory M2 type is essential in the treatment of asthma [72]. MSC-EVs were also found to impact macrophage polarization. One previous study showed that the intravenous administration of human umbilical cord mesenchymal stem cells-derived EVs reversed histopathological changes and lung inflammation in mice asthma model. This therapeutic ability is partly associated with the promoted macrophage M2 polarization by MSC-EVs. In particular, tumor necrosis factor receptor-associated factor 1 (TRAF1) was found to be significantly downregulated in macrophages after treatment with MSC-EVs. This downregulation of TRAF1 further suppressed NF-κB activation and inhibited M1 polarization of macrophages. Meanwhile, the downregulation of TRAF1 promoted the activation of PI3K/AKT for M2 polarization and eventually led to the macrophage transition from M1 type to M2 type [73]. Moreover, MSC-EVs were observed to reduce the proportion of ovalbumin (OVA)-induced M1-type macrophages and enhanced M2-type macrophage activation through MiR-183-5p/FoxO1 signaling axis. As a result, MSC-EVs show a potent capability to downregulate the levels of inflammatory factors in the airways [74].

2.2.4. *Relieving airway remodeling*

The remodeling of the airways is a hallmark feature of asthma, which interacts with airway inflammation in a bidirectional manner and eventually leads to the obstruction of the airways [75]. Previous investigations have demonstrated that MSC-EVs possess the capability to inhibit chronic airway reconstruction during asthma. The critical role and the underlying mechanisms of MSC-EVs in airway remodeling are summarized below. Song et al. showed that MSC-EVs can suppress airway reconstruction and the epithelial-mesenchymal transition through the inhibition of the Wnt/β-catenin signaling pathway. As a result, the intravenous injection of MSC-EVs achieved an efficient inhibition of goblet cell proliferation, as well as a notable reduction of collagen deposition in asthmatic rats [76]. Furthermore, EVs secreted by human bone marrow-derived MSCs were observed to attenuate the anomalous multiplication and migration of bronchial smooth muscle cells (BSMCs), and alleviate collagen deposition and mucus formation in the lung tissues of asthmatic mice [45]. Microarray analyses revealed that this biofunction was closely related to the capability of MSC-EVs in modulating the miR-188/JARID2/Wnt/β-catenin signaling pathway. Additionally, miR-301a-3p in EVs secreted by adipose tissue-derived MSCs was found to be involved in the pathogenesis of asthma: the miR-301a-3p carried MSC-EVs were proficiently internalized by BSMCs, then the miR-301a-3p exhibited significant efficacy in impeding the proliferation and migration of BSMCs. Interestingly, miR-301a-3p was also found to have the ability to decrease the discharge of inflammation-causing agents *via* the downregulation of signal transducer and activator of transcription 3 at the same time. These findings propose that miR-301a-3p in EVs could be a potential therapeutic pathway for attenuating inflammation and airway remodeling in asthma [77]. In addition, hypoxia-primed MSC-EVs (Hypo-EVs) were observed to have a more potent inhibition of lung inflammation and airway reconstruction in asthmatic mice than normoxia-primed MSC-EVs (Nor-EVs). The mechanism is that Hypo-EVs are more effective at lowering the level of pro-fibrogenic markers, including collagen-1, α-smooth muscle actin and TGF-β1-p-smad2/3 signaling pathway. Consequently, they impeded chronic allergic airway restructuring in asthmatic mice. Additionally, Hypo-EVs exhibited marked enrichment of miR-146a-5p compared to Nor-EVs, enabling a more efficient inhibition of inflammation and mitigation of fibrosis. The above results further confirmed the crucial capability of MSC-EVs in suppressing airway remodeling to relieve asthma and demonstrated the potential of hypoxic preconditioning in increasing the therapeutic efficiency of MSC-EVs against asthma [78].

2.2.5. *Other possible mechanisms*

The aforementioned summary delineates the diverse modalities by which MSC-EVs primarily mediate their therapeutic effects in the management of asthma. Numerous investigations have been explored to figure out the underlying mechanisms of how EVs affect recipient cells, such as the activation of various signaling pathways and the up- or down-modulation of soluble cytokines. Interestingly, almost all the current studies indicate that miRNAs in EVs play a dominant role in the management of asthma. Meanwhile, as far as we know, the therapeutic potential of other contents in EVs, like cytokines, chemokines, mRNA, lipids or metabolites, in the treatment of asthma are rarely reported. However, some of these contents in EVs have been demonstrated to assist in the inhibition of inflammation in other disease models. For example, chaperonin containing TCP1 subunit 2 in MSC-EVs was reported to attenuate inflammatory cell infiltration by suppressing CD154 synthesis in CD4+ *T* cells [79]. Hence, further studies to figure out the contributions of other contents in addition to miRNA in EVs in the treatment of asthma are of great significance. Such investigations not only expand our understanding of the therapeutic mechanism of EVs but also shed light on new targets involved in the pathologic process of asthma.

3. Delivery approaches of MSC-EVs

MSC-EVs have shown bright prospects in preclinical models of asthma, but optimal delivery approaches remain to be investigated. In particular, the administration approaches of EVs have certain impacts on the biodistributions and bioavailability of administrated MSC-EVs, which is essential for both the improvement of therapeutic efficiency and the reduction of potential side effects. Therefore, the advantages and disadvantages of different administration approaches of EVs are discussed with detailed examples in the following. Thus far, three primary administration strategies have been employed to deliver EVs for asthma treatment: intravenous administration, intratracheal drip and nebulized inhalation. A short comparison of these three administration strategies is provided in Table 1. Of note is that the intravenous administration of MSC-EVs for asthma treatment is currently dominant. However, pioneer studies have shown the promise of pulmonary administration of EVs for treating asthma and other pulmonary diseases. In the following sections, both the intravenous and pulmonary administrations of EVs will be briefly introduced and discussed. Considering the treatment of asthma using pulmonary administrated EVs is still at an early stage, several examples of using EVs to treat other pulmonary diseases are also summarized to indicate the potential usage of the corresponding delivery strategy in asthma treatment.

3.1. *Intravenous injection*

Intravenous injection is currently the most applied approach for the administration of EVs, showing the advantages of being free of additional equipment, precise dose control and relatively low cost. A previous study has demonstrated that the intravenous administration of MSC-EVs achieves a successful reduction of the inflammatory level in the asthmatic mouse model [78]. However, the intravenous approach has its inherent weaknesses, including the invasive administration, the fast clearance of administrated EVs from circulation, and the relative low efficiency for airways targeting [80]. Generally, the intravenous administrated EVs are initially distributed in the spleen, the kidney, the liver, and the lung at approximately 30 min after the injection.

Table 1 – Comparison of different delivery routes of EVs [91–94,99,119].

Delivery routes	Advantages	Disadvantages
Intravenous injection	Additional equipment free; Precise dose control; Well-proven clinically	Invasive administration; Fast clearance from circulation; Low efficiency of airways targeting
Pulmonary administration	Lung restricted distribution; Good bioavailability; Low safety risks to other organs	Delivery efficiency and lung deposition are easily affected by impaired lung function
Intratracheal instillation	Precise dosage control	Uneven drug distribution
Nebulized inhalation	Even drug distribution; Non-invasive	Relatively difficult to precisely control the administrated dosage between individuals

In addition, EVs show the highest distribution in the spleen, partly due to their vascular aggregation [81]. However, this distribution is just a regular result. In fact, EVs with different sizes exhibit distinct biodistribution and retention characteristics. According to the guidelines provided by the International Society for Extracellular Vesicles, EVs can be split into two main classes based on their size: small EVs (sEVs), typically measuring less than 100 nm, and large EVs (lEVs), typically measuring more than 200 nm [57]. sEVs were observed to mainly distribute in the liver after intravenous administration and presented a slow decline from the liver (could be observed until 72 h) [82]. Meanwhile, the accumulation of sEVs in the lung was 5–6 times lower than that in the liver and did not reach peak level until 2 h after the initial administration [83–85]. By contrast, lEVs demonstrated a quick accumulation in the lung after intravenous administration and thereafter declined within 2–12 h, but their distributions in the liver lasted a longer time (over 24 h) [86]. These results indicated that the particle size of EVs has a pivotal impact on the biodistribution of intravenously administrated EVs: the lEVs prefer to distribute in the lung, and sEVs tend to accumulate in the liver. Hence, such findings also imply that only a partial of the EVs are distributed the lungs through intravenous administration. In addition to the particle size, the surface protein on EVs was reported to impact the pulmonary distribution. For example, integrin $\alpha_6\beta_1$ on EVs is beneficial for the pulmonary distribution of EVs [87]. Furthermore, the rapid elimination of EVs *via* intravenous administration is another challenge to achieve a sufficient retention of EVs in the bronchi and the lungs. According to the previous findings, 95 % of the intravenously administrated EVs were eliminated from circulation within 5 min after the injection [82,88,89], and the half-life of EVs after intravenous injection was reported to be roughly 2–4 min [90]. Therefore, the relative low delivery efficiency to the lungs and the airways, in addition to the short half-life of EVs after intravenous injection, are urging for efforts to find more efficient delivery approaches of EVs for the treatment of respiratory disease.

3.2. *Pulmonary administration of EVs*

Compared with the intravenous administration of EVs, the local administration to the lungs is a more direct and efficient delivery strategy. Generally, lung tissues are comprised of bronchi, small bronchi, alveolar ducts, alveoli, and pulmonary blood vessels. For asthma treatment, bronchi are the major target for the delivery of medicants [75]. The pulmonary administration takes advantage of directly transporting the therapeutic agent to the bronchi, meanwhile decreasing the risks of entering circulatory system and the off-target distribution [91]. Consequently, pulmonary administration improves the bioavailability of EVs and reduces risks of adverse reactions [92]. Despite the demonstrated superiority of pulmonary administration, the direct delivery of medicines to lung tissues through the pulmonary pathway remains challenging. The bronchoconstriction, excessive mucus secretion, and airway remodeling in the injured lungs may lead to a low delivery efficiency and a temporary retention of drugs in the diseased lungs [93,94]. Currently, intratracheal instillation and aerosol inhalation are the two primary methods for pulmonary delivery of EVs.

3.2.1. *Intratracheal instillation*

The possibilities of EVs administration through intratracheal instillation have been studied in the treatment of asthma, pulmonary fibrosis, and bronchial dysplasia [95–97]. For example, the intratracheal instillation of MSC-EVs was demonstrated to successfully promote the polarization of macrophages from M1 type to M2 type, and reversed the AHR, as well as attenuated the inflammation response, resulting in an efficient control of asthma [73]. The intratracheal administration of MSC-EVs also showed an efficient alleviation of airway inflammation by inhibiting the apoptosis in pulmonary niche, thus significantly ameliorating the pathological changes in asthmatic rats [98]. Generally, the delivery strategy using intratracheal instillation takes significant advantage of precise control of the administrated dosage, which is largely due to the direct dripping of drugs into the trachea [99]. Nevertheless, compared to nebulizers or inhalers, intratracheal instillation is a more invasive delivery strategy, setting inconvenience for their potential clinic applications [100]. In addition, intratracheal instillation has relatively limited lung dispersion, which may lead to uneven drug distribution [101].

3.2.2. *Nebulized inhalation*

Nebulized inhalation is another most applied pulmonary administration strategy, which involves the aerosol generators to enable a targeted delivery of therapeutic components directly to the lungs for a rapid onset of action. Presently, there are three primary types of aerosol generators: pressurized metered dose inhalers, dry powder inhalers, and medical

nebulizers [102]. In experimental research, nebulizers are commonly utilized as atomization equipment, including jet nebulizer (air compression nebulizer), ultrasonic nebulizer, and vibrating mesh nebulizer (VMN) [103]. Among them, the ultrasonic nebulizer faces the problem of heat generation, which may cause thermal inactivation of drugs and are not suitable for delivering EVs [104]. The jet nebulizer atomizes the liquid solution by compressing the gas; this mechanism possesses an obvious shortage of large amounts of drug residue in the device [105]. Considering this weakness may cause a large waste of valuable EVs, jet nebulizer is also not the best option for the EVs administration. At last, VMN is a recently developed nebulizer that vibrates an orifice plate with holes of uniform size. This equipment overcomes the shortages of the above two mentioned nebulizers, showing superiorities of a uniform dose administration and minimal drug residue after administration [106]. One of the previous studies has compared the delivery efficiency between VMN and jet nebulizer, showing six-fold increased lung aerosol deposition using VMN than that using jet nebulizers [107]. In addition, VMN can avoid fugitive emissions of therapeutic agent aerosols, taking advantage of minimal risks for clinical applications [108–110].

Several pioneer studies have investigated the potential of utilizing nebulizers to deliver MSC-EVs for treating pulmonary ailments. For instance, some previous studies reported the success of using air compression nebulizers for an efficient pulmonary administration of EVs [111,112]. Furthermore, another study has demonstrated that the sEVs isolated from mouse serum can be efficiently delivered to lipopolysaccharide-injured lungs and could enable long-term lung retention over 5 days by using VMN (Fig. 4). This study also demonstrated that the inhaled EVs were mainly taken up by pulmonary macrophages and airway epithelial cells [113]. Thus far, nebulized inhalation of MSC-EVs for asthma treatment is still in the early validation stage. A recent study has shown that nebulized inhalation possesses great potential as a non-invasive route for the administration of MSC-EVs in asthma treatment. In this work, researchers collected the EVs from hypoxic preconditioned MSCs and administrated these hypoxic EVs by atomizing inhalation (Fig. 5). The nebulization was observed to have no adverse impacts on the structural integrity of EVs. In addition, the administration using inhalation showed an efficient and long-term (7 d) restriction of inhaled MSC-EVs in mice lungs, resulting in a potent attenuation of allergic airway inflammation and remodeling [114]. Of note, previous observation has found that the nebulized inhalation of EVs derived from lung spheroid cells and human embryonic kidney cells at same doses showed similar lung distribution, but EVs derived from lung spheroid cells possessed a better lung deposition [115]. This finding implied the fact that the origin of EVs may impact the lung deposition of inhaled EVs. Therefore, further investigations of MSC-EVs derived from different types of MSC or different preconditioned MSC can be an interesting research subject and may assist in the improvements of administration efficiency of MSC-EVs *via* inhalation.

Furthermore, the safety and efficacy of aerosolized inhalation of MSC-EVs have been preliminarily verified in a clinical trial. The EVs derived from adipose-derived MSCs were administrated to seven patients with severe COVID-19-associated pneumonia through VMN. Results showed that the patients tolerated inhaled dosages of up to 2.0×10^9 clinical-grade MSC-EVs for 5 days in a row without experiencing any unexpected side effects, and all patients saw varied degrees of pulmonary lesion reduction [116]. However, additional studies in enlarged populations and evaluations of long-term safety are required for the potential clinic translation of using an inhalation approach to deliver MSC-EVs for treating pulmonary diseases, including asthma.

3.3. *Intravenous or pulmonary administration?*

As summarized above, compared with intravenous administration, pulmonary administration appears advantageous for more efficient lung delivery of EVs and their deposition throughout the bronchi and epithelium [117,118]. Especially, the inhalation strategy possesses the obvious superiority of a non-invasive administration of EVs and minimal risks to induce systemic toxicity [119]. Currently, the direct comparison of intravenous and pulmonary administrations of EVs for asthma treatment is lacking. But there is a study that compared the lung targeting efficiency of administrating MSC-EVs using intravenous injection and aerosol inhalation in the mouse model of acute lung injury (ALI). Therapeutic results showed that the inhalation strategy achieved a more efficacious result in inhibiting lung inflammation and protecting lung cells from ALI injury than the intravenous administration [120]. Additionally, Yang et al. investigated the biodistribution of delivering EVs through intravenous and intratracheal routes. Their results showed that most intravenous administrated EVs were accumulated in the liver and the spleen, whereas intratracheal administration demonstrated the advantages of a prolonged retention of EVs in the diseased lung. Notably, this study further revealed that the administration route of EVs not only impacted the biodistribution of EVs, but also determined their therapeutic mechanism [95]. This finding highlighted the importance of the administration route of EVs in pulmonary disease treatment and provided a potential strategy to augment the therapeutic capability of EVs by altering their administration route.

From our opinion, the inhalation of MSC-EVs for asthma treatment can be an attractive strategy, but there are still several challenges requiring to overcome. For example, variations in deposition patterns due to stochastic changes in airway geometry between individuals are of particular concern. This inter-subject variation in deposition may result in compromised drug inhalation efficiency [121]. A typical example is the obese individuals, who usually demonstrate narrow airways [122]. In addition, the injured lung function of patients may also adversely affect the aerosol deposition of inhaled drugs, which is mainly due to airway remodeling in asthma [123]. Bronchoconstriction and excessive mucus production caused by the airway remodeling may lead to airway obstruction, thereby decreasing the delivery efficiency to bronchi [93,94]. Therefore, future studies to enhance the stability, administration efficiency and biosafety of inhalation strategy will be intriguing and valuable to guide the possible

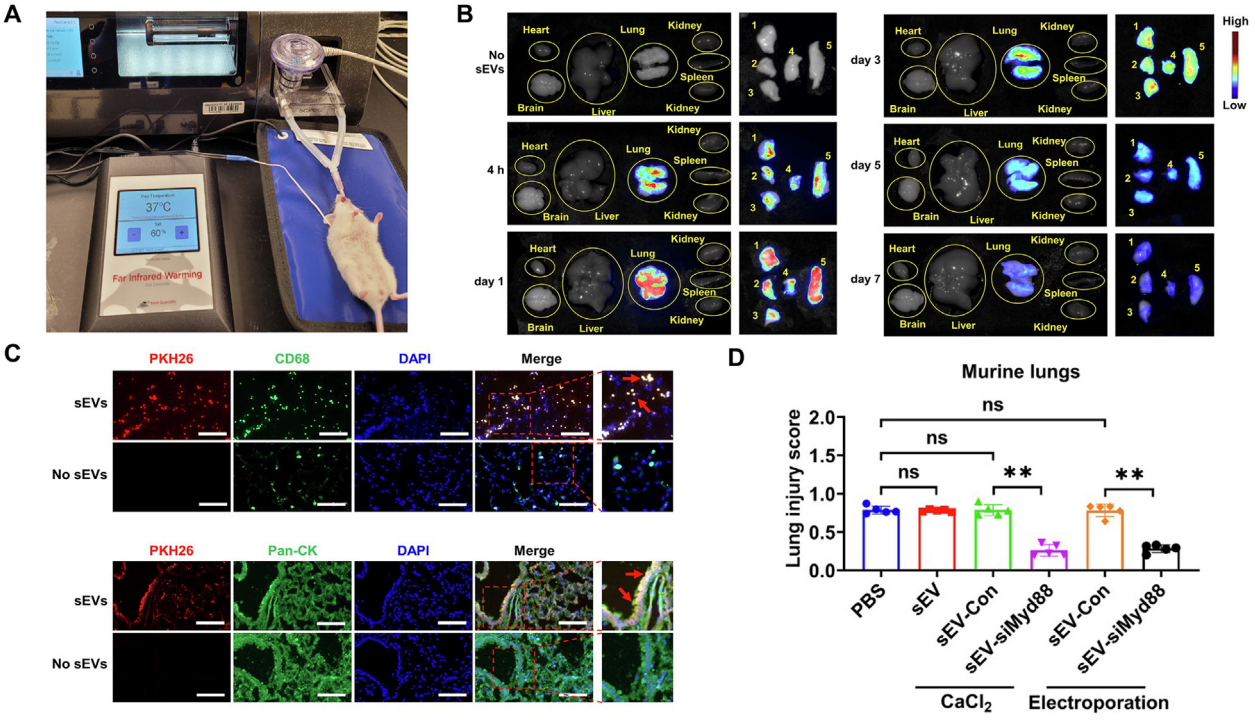

Fig. 4 – Aerosolized sEVs from mouse serum were used as a carrier to deliver small RNAs to treat lung injury. (A) The mouse in the image is being treated with sEVs *via* Aeroneb VMN after endotracheal intubation. (B) Representative images showing organ distribution and fluorescence intensity at indicated time points after inhalation of DiR-tagged sEVs in mice. Negative control is the DiR-tagging step performed without EVs. (C) The biodistribution of inhaled sEVs in mouse lungs. PKH26-tagged sEVs were administrated by aerosol, and their detailed distribution in lung sections was examined using immunofluorescence staining. Macrophages were indicated by antibodies against CD68, and lung epithelial cells were indicated by antibodies against Pan-CK. (D) Lung injury scores corresponding to each group are shown in histograms: $n = 5$. Results are mean ± SD and analyzed by a one-way ANOVA with the Tukey method. ns, $P > 0.05$, $^{**}P < 0.01$. Reproduced with permission from [113]. Copyright 2022 Elsevier B.V.

application of administrating MSC-EVs through inhalation for an efficient and convenient control of asthma.

4. Prospect

The current research has shown the bright promise and unique advantages of MSC-EVs in asthma treatment, which are mainly owing to their immunomodulation capability. Further studies indicate that this capability is closely associated with the contents in MSC-EVs, especially the different types of miRNAs. However, the detailed molecular mechanisms underlying the diverse miRNAs in EVs are still not fully understood, warranting further investigations into the molecular mechanisms involved in EV-mediated asthma treatment. A previous study has revealed that MSC-EVs could significantly ameliorate immune destruction of hematopoietic stem cells in the aplastic anemia mouse model. The therapeutic mechanism is closely related to the inhibition of IFN-γ and TNF-α production, which is caused by the miR-199a, miR-146a, miR-223, and miR-126 in MSC-EVs [124]. This finding may provide a possible explanation for the immune suppression of using MSC-EVs in asthma treatment. Further efforts to figure out the underlying mechanism are important, because these findings will provide the crucial theoretical foundation for further pretreatments or modifications of EVs to improve their curative efficiency against asthma [125]. In addition, the administration routes of EVs have been demonstrated to have a significant impact on the biodistribution of EVs, thereby determining the therapeutic efficiency or the therapeutic mechanism. Nebulized inhalation of EVs has demonstrated superiority in the treatment of lung disease in animal studies, suggesting a potential administration strategy to deliver EVs. In fact, this delivery approach has been successfully used in the vaccine delivery targeting the lungs [126]. Moreover, clinical trials for the treatment of COVID-19 (NCT04602442, NCT04276987), acute respiratory distress syndrome (NCT04602104), and pulmonary infection (NCT04544215) also confirmed the promising potentials of nebulized inhalation in the clinical applications. Therefore, further efforts to optimize the delivery of aerosolized EVs through inhalation are necessary to set this delivery strategy into practical applications [127].

Regarding the clinical application of using MSC-EVs for asthma management, there is still a long way and full of challenges: (1) The quality control of collected MSC-EVs, especially regarding the content in EVs, is still a major

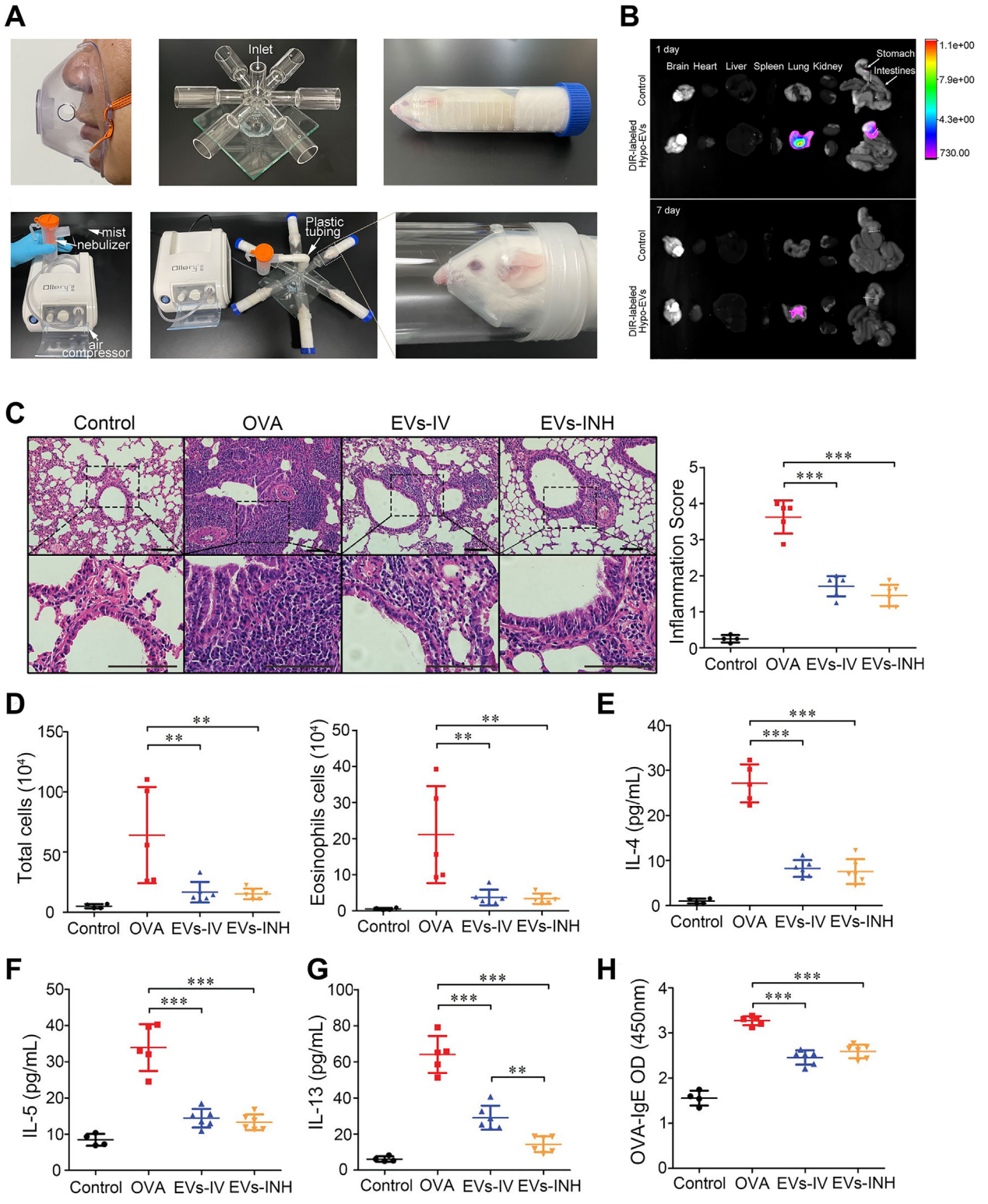

Fig. 5 – Administration of hypoxic MSC-EVs *via* nebulized inhalation for treating asthmatic airway inflammation and remodeling. (A) Illustration of the atomization inhalation system with six-pass inhalation chamber for mice. This system can simulate the nebulized inhalation mask used in humans. (B) Inhalation of nebulized MSC-EVs showed an efficient and long-term (7 d) accumulation in the lungs. (C) Representative images of lung sections stained using H&E after indicated treatments, Scale bar = 100 μm (left panel). The inflammatory infiltration of the lung samples was evaluated by the calculation of inflammation score according to the H&E staining images (right panel): $n = 5$. (D) Number of the total inflammatory cells and eosinophils in BALF. (E–G) Levels of IL-4, IL-5 and IL-13 in BALF: $n = 5$. (H) OVA-specific IgE levels in serum: $n = 5$. $^{**}P < 0.01$, $^{***}P < 0.001$. Reproduced with permission from [114]. Copyright 2023 Frontiers Media S.A.

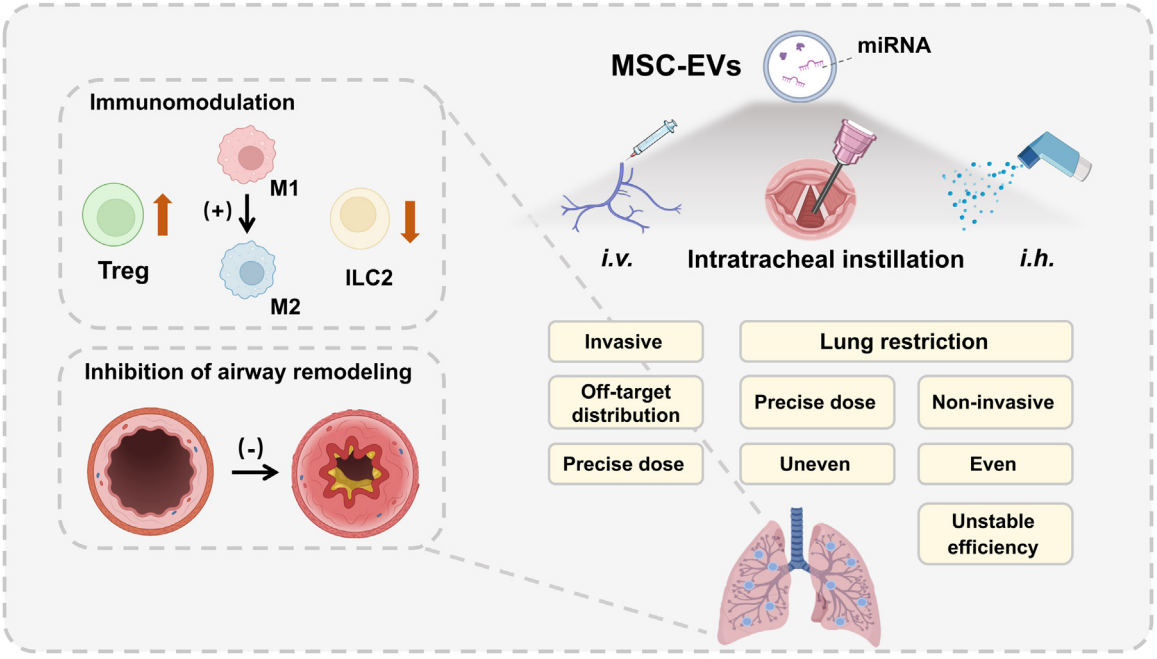

Fig. 6 – Illustration summary of using MSC-EVs for asthma treatment. MSC-EVs can be a novel cell-free agent for asthma control due to their capacity for immunoregulation and airway remodeling inhibition. Diverse miRNAs in MSC-EVs participate in the alleviation of asthma symptoms. The delivery routes also have impacts on the therapeutic efficiency of using MSC-EVs to treat asthma. Intravenous injection (*i.v.*) is currently the most used strategy for delivering EVs, facing challenges of relatively low delivery efficiency to the airways and undesired accumulations in other organs. Meanwhile, pulmonary administration, including intratracheal instillation and nebulized inhalation (*i.h*), shows advantages in efficient delivery of EVs to the airways with minimal invasive. Especially, nebulized inhalation of MSC-EVs is currently regarded as a promising strategy for asthma treatment.

challenge. (2) More investigations about the potential risks, especially the long-term safety risks of using MSC-EVs to treat asthma, are demanded. (3) Aerosol-based inhalation provides a feasible and non-invasive delivery method for EVs administration, but the precise control of the administrated dose of EVs, as well as the consistency between different individuals, remain a challenge. (4) The mass production of MSC-EVs in clinical grade with a good homogeneity also sets a huge challenge for the therapeutic use of MSC-EVs. Similar to the production of therapeutic stem cells, a Good Manufacturing Practice (GMP) for MSC-EVs production is necessary. (5) In addition to the EVs production, the storage and transportation of EVs are also critical. Further developments of novel technologies to improve the satiability of EVs are another important field. Overcoming the mentioned challenges and other potential obstacles may promote the therapeutic applications of MSC-EVs on the clinical bench.

5. Conclusion

The present review provides a comprehensive overview of using MSC-EVs as novel therapeutic agents to treat asthma. As summarized above, EVs play vital roles in mediating the immunomodulation and anti-inflammation functions of MSCs. Therefore, they have the potential to replace MSCs as a cell-free treatment for controlling asthma by impacting the immune cells and inhibiting airway remodeling. In addition, the administration routes of EVs also have significant impacts on the therapeutic efficiency against asthma. Pulmonary delivery takes inherent advantage of direct lung delivery of EVs for local administration in comparison with the commonly used intravenous administration. Among all pulmonary delivery routes, nebulized inhalation using VMNs holds the advantages of a uniform dose administration and an effective delivery of EVs, which can be a promising strategy for a non-invasive and highly efficient administration of EVs for asthma treatment (Fig. 6). However, there are still lots of barriers during the translation of MSC-EVs to medical practices. With the continued efforts to overcome the limitations of EVs in therapeutic applications, such as mass production, quality control of production, and systemic and long-term safety evaluations, these cell-derived vesicles can be a potential option to satisfy the asthma treatment.

Declaration of competing interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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