Received: 29 June 2023 | Revised: 1 March 2024 | Accepted: 15 March 2024

DOI: 10.1111/all.16117

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI WILEY

ORIGINAL ARTICLE

Drug Allergy, Insect Sting Allergy and Anaphylaxis

Extracellular vesicle miRNAs drive aberrant macrophage responses in NSAID-exacerbated respiratory disease

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Funding information
Helmholtz Initiative and Networking; Deutsche Forschungsgemeinschaft; Else Kröner-Fresenius-Stiftung

Abstract

Background: Extracellular vesicles (EVs) have been implicated in the pathogenesis of asthma, however, how EVs contribute to immune dysfunction and type 2 airway inflammation remains incompletely understood. We aimed to elucidate roles of airway EVs and their miRNA cargo in the pathogenesis of NSAID-exacerbated respiratory disease (N-ERD), a severe type 2 inflammatory condition.

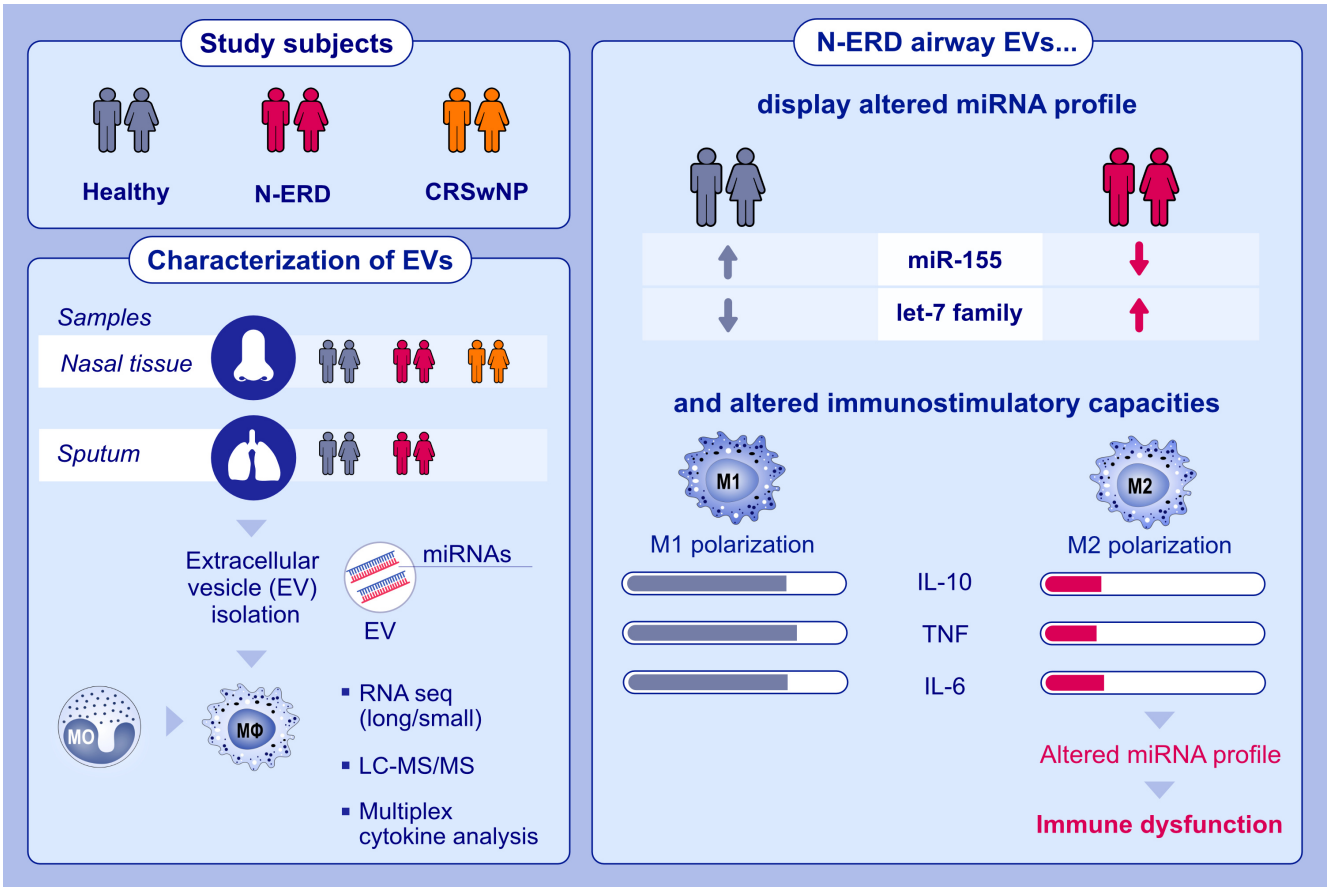
Methods: EVs were isolated from induced sputum or supernatants of cultured nasal polyp or turbinate tissues of N-ERD patients or healthy controls by size-exclusion chromatography and characterized by particle tracking, electron microscopy and miRNA sequencing. Functional effects of EV miRNAs on gene expression and mediator release by human macrophages or normal human bronchial epithelial cells (NHBEs) were studied by RNA sequencing, LC–MS/MS and multiplex cytokine assays.

Results: EVs were highly abundant in secretions from the upper and lower airways of N-ERD patients. N-ERD airway EVs displayed profoundly altered immunostimulatory capacities and miRNA profiles compared to airway EVs of healthy individuals. Airway EVs of N-ERD patients, but not of healthy individuals induced inflammatory cytokine (GM-CSF and IL-8) production by NHBEs. In macrophages, N-ERD airway EVs exhibited an impaired potential to induce cytokine and prostanoid production, while enhancing M2 macrophage activation. Let-7 family miRNAs were highly enriched in sputum EVs from N-ERD patients and mimicked suppressive effects of N-ERD EVs on macrophage activation.

Conclusion: Aberrant airway EV miRNA profiles may contribute to immune dysfunction and chronic type 2 inflammation in N-ERD. Let-7 family miRNAs represent targets for correcting aberrant macrophage activation and mediator responses in N-ERD.

KEYWORDS
asthma, CRSwNP, extracellular vesicles, macrophages, type 2 immunity

Franziska Hartung and Pascal Haimerl contributed equally.



GRAPHICAL ABSTRACT

EVs isolated from the upper and lower airways of N-ERD patients display altered miRNA profiles compared to healthy controls. Small RNA sequencing reveals upregulation of let-7 miRNAs and downregulation of miR-155 in N-ERD sputum EVs. Let-7 family miRNAs promote macrophage M2 activation, while limiting EV-triggered cytokine responses, thus implicating aberrant EV miRNA profiles in N-ERD pathogenesis. Abbreviations: CRSwNP, chronic rhinosinusitis with nasal polyps; EV, extracellular vesicle; IL-interleukin; LC-MS/MS, liquid chromatography-mass spectrometry; miRNA, microRNA; MO, monocyte; Mϕ, macrophage; N-ERD, nonsteroidal antiinflammatory drug-exacerbated respiratory disease; TNF, tumor necrosis factor.

1 | INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAID)-exacerbated respiratory disease (N-ERD) is a chronic type 2 inflammatory disease of the upper and lower airways. N-ERD represents a particularly severe subtype of chronic rhinosinusitis with nasal polyps (CRSwNP) which affects 9.7% of CRSwNP patients.[1] Symptoms of CRSwNP include nasal congestion, hyposmia, and rhinorrhea.[2,3] N-ERD patients suffer from CRSwNP, asthma and intolerance to NSAIDs.[4] Recent work has shown that monocyte-derived macrophages (MDM) from N-ERD patients exhibit aberrant transcriptional profiles and mediator responses as well as reduced DNA methylation compared to MDM from healthy controls.[5] Macrophages are potent producers of eicosanoids, arachidonic acid (AA)-derived lipid mediators with key functions in type 2 immune responses.[6] The severe type 2 inflammation in N-ERD is perpetuated by a defect in the generation of regulatory cyclooxygenase (COX) metabolites (prostanoids) such as prostaglandin E_2 (PGE_2) as well as an overproduction of cysteinyl leukotrienes (cysLTs) and type 2 cytokines.[7,8]

Extracellular vesicles (EVs) have been implicated in airway inflammation and the generation of pro-inflammatory eicosanoids.[9,10] EVs can be classified, depending on their size and origin, into exosomes (30–100 nm), microvesicles (100–1000 nm) and apoptotic bodies (>1 μm).[11,12] Exosomes and microvesicles are derived from the endosomal membrane forming multivesicular bodies or the plasma membrane, respectively. EVs carry various lipids, proteins and RNAs, and immunoregulatory functions of EVs have particularly been attributed to their microRNA (miRNA) cargo.[13,14] Several studies have shown an altered EV concentration and cargo in bronchoalveolar lavage fluid (BALF), nasal lavage, and serum of asthmatics.[10,15–19] However, a potential involvement of EVs in the pathogenesis of N-ERD has remained unknown.

We isolated EVs from the upper and lower airways of N-ERD patients at high purity by size-exclusion chromatography[20] and performed small (miRNA) and long (mRNA) RNA sequencing as well as targeted lipidomics to characterize the miRNA cargo and immunological effects of N-ERD EVs. The miRNA cargo of sputum as well as nasal tissue-derived EVs in N-ERD was distinct from NSAID tolerant individuals and characterized by an enhanced abundance of let-7 family members and reduced miR-155-5p levels, known to regulate inflammation and macrophage polarization. Compared to EVs from the airways of healthy individuals, N-ERD EVs showed a strongly

reduced immunostimulatory capacity, which could be mimicked by let-7 miRNAs. Thus, EV miRNAs may possess biomarker potential and represent a therapeutic target for the treatment of infection-induced exacerbations in N-ERD.

2 | MATERIALS AND METHODS

A detailed description of the methods used in this study is provided in this article's Online Repository.

2.1 | Study approval

This study was approved by the local ethics committee at the Klinikum rechts der Isar, Technical University of Munich (internal reference: 422/16). Written informed consent in accordance with the Declaration of Helsinki was obtained from all patients.

2.2 | Patient characterization

N-ERD and NSAID tolerant (NT) CRSwNP patients and healthy controls were recruited to the Klinikum rechts der Isar (Munich, Germany). The same subjects were used in a previous study[5] presenting detailed patient information and clinical characteristics (Table S1).

2.3 | Isolation of sputum and nasal tissue-derived extracellular vesicles

Sputum induction of healthy individuals and N-ERD patients was performed as previously described.[21] Healthy turbinate tissue or nasal polyp tissue was cultured at air-liquid interface for 24 h. EVs were isolated from sputum and tissue supernatant (SN) by precipitation and purified by size-exclusion chromatography (SEC) according to the guidelines.[22]

2.4 | Alveolar-like monocyte-derived macrophage culture

$CD14^+$ monocytes were isolated from healthy, CRSwNP and N-ERD subjects and differentiated into alveolar-like monocyte-derived macrophages (MDM) as previously described.[23–25]

2.5 | RNA isolation and real-time quantitative PCR (qPCR)

RNA isolation and qPCR was performed as previously described.[25] Small and large RNAs were purified according to the manufacturer's instructions (Zymo Research).

2.6 | Lipid mediator analysis

Targeted lipidomics was performed by LC–MS/MS as previously described.[26]

3 | RESULTS

3.1 | EVs are abundant in sputum and nasal polyp tissue of patients suffering from CRSwNP and N-ERD

Previous studies suggested that EVs in the airways may contribute to the pathogenesis of allergic asthma.[10] Thus, we investigated whether EVs are present in the upper and lower airways of N-ERD patients. We isolated EVs from induced sputum or tissue culture supernatants of nasal polyp or turbinate tissues from N-ERD and NSAID tolerant (NT) CRSwNP patients or healthy controls by size-exclusion chromatography (SEC).[22] Nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) confirmed the presence of EVs ranging from 50 to 850 nm corresponding to the expected size range of exosomes and microvesicles (Figure 1A–D, Figure S1A), but not apoptotic bodies. EV yield was similar for N-ERD (8.72×10^8 particles/mL) and healthy (8.85×10^8 particles/mL) sputum. TEM imaging suggested that EVs were more abundant in nasal polyp tissue as compared to turbinate tissues from healthy individuals (Figure 1D, Figure S1A). This suggests that EVs are highly abundant in the upper and lower airways of N-ERD patients and may thus contribute to the immune dysregulation and type 2 inflammation driving CRSwNP and bronchial asthma in N-ERD.

3.2 | Lower airway EVs from N-ERD patients show a reduced capacity to activate macrophages

Macrophages are key players in inflammation and host defense in the airways and aberrant macrophage function has recently been implicated in the pathogenesis of N-ERD.[5] Thus, we performed RNA sequencing (RNAseq) to study whether airway EVs from N-ERD patients and healthy controls may differentially modulate macrophage activation. Human monocyte-derived macrophages (MDM) were isolated from healthy donors and differentiated with TGFβ and GM-CSF mimicking the airway cytokine milieu.[26] MDM were treated with sputum EVs isolated from healthy individuals or N-ERD patients for 24 h prior to RNAseq (Figure 2A). Five hundred and twenty-four genes were differentially expressed between MDM exposed to healthy versus N-ERD airway EVs (Table S2). Compared to mock control (qEV PBS), exposure to healthy and N-ERD EVs induced the expression of pro-inflammatory genes including *CXCL5*, *CCL8*, *IL12B*, *IL6*, *IL19*, *IL1B*, *S100A12*, and *CCL2* (Figure 2B,C, Table S2). However, the transcription of inflammatory and host defense genes was more prominently induced by healthy as compared to N-ERD EVs, suggesting that N-ERD airway EVs display an impaired capacity to trigger pro-inflammatory host defense

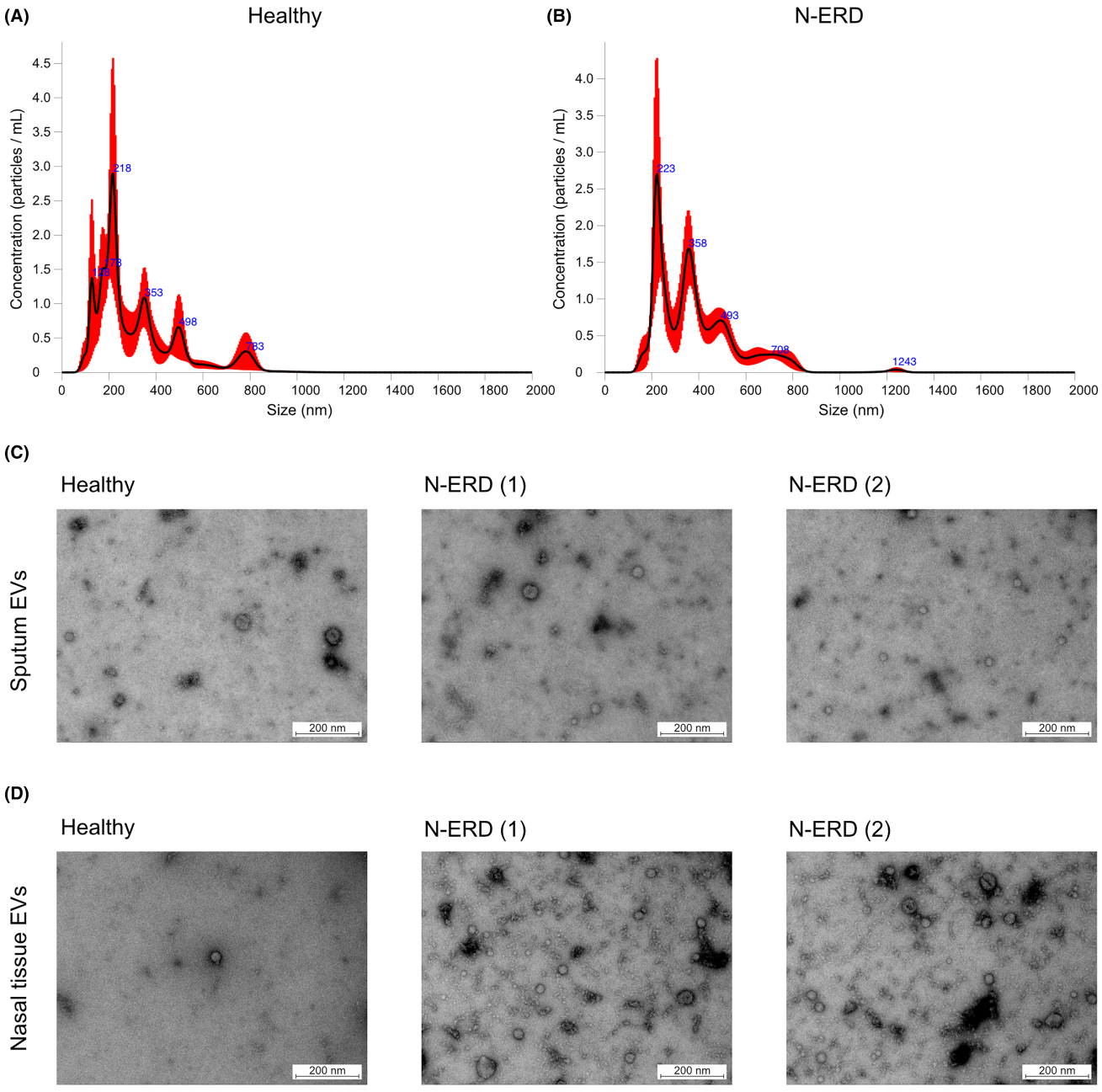
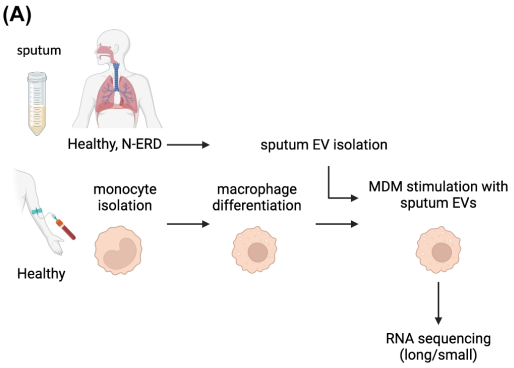
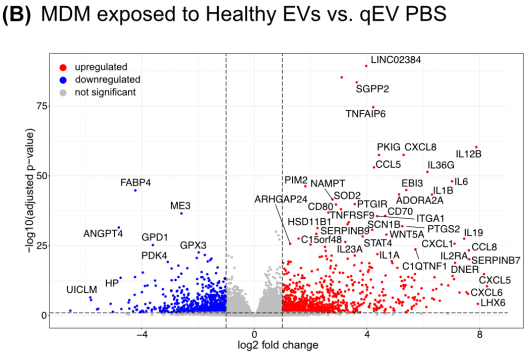
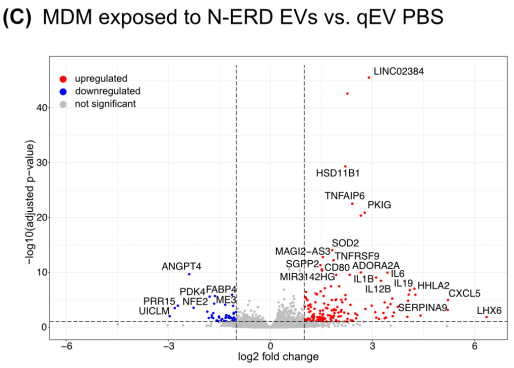


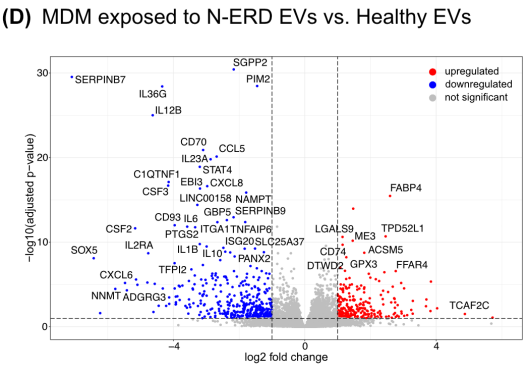
FIGURE 1 EVs are abundant in sputum and nasal polyp tissue of patients suffering from NSAID-exacerbated respiratory disease. (A, B) Nanoparticle tracking analysis (NTA) of EVs isolated from induced sputum from healthy individuals ($n = 10$) (A) or N-ERD patients ($n = 5$) (B), concentration distributions and particle size were measured in triplicates, SEM is shown in red; (C, D) Transmission electron microscopy (TEM) images showing representative EV preparations from induced sputum (C) or nasal turbinate or polyp tissue culture supernatant (D) from healthy subjects or patients suffering from N-ERD.

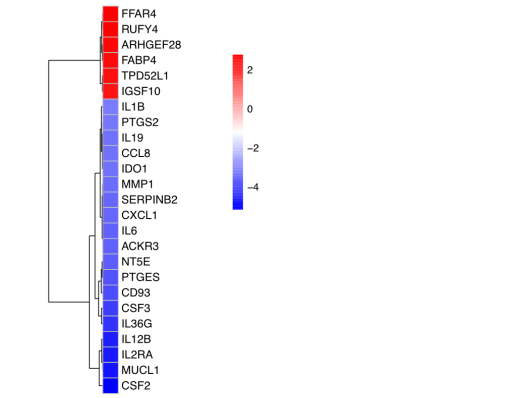
FIGURE 2 Exposure to airway EVs from N-ERD patients limit inflammatory response of macrophages. (A) Experimental design of EV isolation and stimulation of MDM with sputum EVs for 24 h, created with BioRender.com; (B–H) RNAseq data for MDM ($n = 6$) stimulated with EVs isolated from sputum of N-ERD patients or healthy individuals: Volcano plot (B, C) of differentially expressed genes (DEG) (base mean >100, $\log_2$ fold change ≥1 or ≤−1, respectively, adjusted p-value ≤.1) of MDM exposed to healthy EVs (B) or N-ERD EVs (C) compared to PBS; Volcano plot (D) and heatmap (E) of top 19 differentially expressed genes and top six genes with the highest positive fold change between MDM exposed to N-ERD sputum EVs vs. Healthy sputum EVs; (F, G) KEGG pathway analysis of MDM exposed to healthy EVs (F) or N-ERD EVs (G) compared to PBS; (H) KEGG pathway analysis of MDM exposed to N-ERD sputum EVs vs. Healthy sputum EVs. Samples are pooled from two individual experiments.

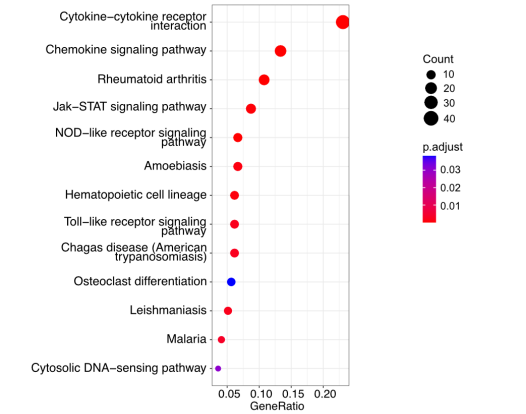

(A)
sputum
Healthy, N-ERD
sputum EV isolation
monocyte isolation
macrophage differentiation
MDM stimulation with sputum EVs
Healthy
RNA sequencing (long/small)

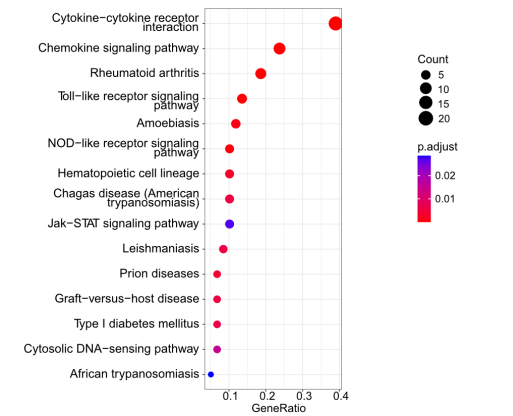

(B) MDM exposed to Healthy EVs vs. qEV PBS
upregulated
downregulated
not significant
−log10(adjusted p−value)
log2 fold change

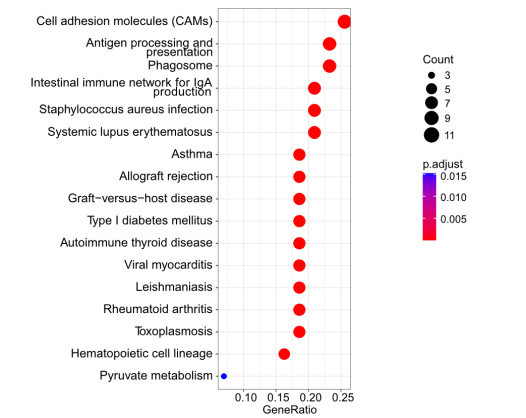

(C) MDM exposed to N-ERD EVs vs. qEV PBS
upregulated
downregulated
not significant
−log10(adjusted p−value)
log2 fold change


(D) MDM exposed to N-ERD EVs vs. Healthy EVs
upregulated
downregulated
not significant
−log10(adjusted p−value)
log2 fold change


(E) MDM exposed to N-ERD EVs vs. Healthy EVs


(F) MDM exposed to Healthy EVs vs. qEV PBS
Count
p.adjust
GeneRatio


(G) MDM exposed to N-ERD EVs vs. qEV PBS
Count
p.adjust
GeneRatio


(H) MDM exposed to N-ERD EVs vs. Healthy EVs
Count
p.adjust
GeneRatio

functions of macrophages. Compared to healthy EVs, N-ERD EVs elicited a reduced induction of genes associated with the suppression of type 2 immune responses (*IL12B*, *EBI3*, *PTGS2*, and *IL10*).[27–30] *FABP4* and several MHC and MHC-related genes (e.g., *HLA-DPB1*, *HLA-DPA1*, *HLA-DRB1*, *CD1A*, *CD1B*, and *CD1C*) were more prominently downregulated in response to healthy as compared to N-ERD EVs (Figure 2D,E, Table S2), indicative of distinct capacities to regulate T-cell responses. KEGG enrichment analysis revealed that both healthy and N-ERD airway EV exposure induced multiple cytokine and chemokine pathways in MDM (Figure 2F,G), while pathways associated with asthma, *S. aureus* infection and antigen presentation were enriched in MDM stimulated with sputum EVs from N-ERD patients (Figure 2H).

In contrast to mRNA sequencing, small RNA sequencing (miRNAseq) of MDM exposed to sputum EVs revealed only minor changes (Figure S2A–C, Table S2). Intriguingly, exposure to N-ERD EVs showed a stronger impact on MDM miRNA profiles compared to exposure to healthy EVs, suggesting that N-ERD sputum EVs may transport altered or enhanced levels of miRNAs to target cells, potentially contributing to their aberrant immunostimulatory potential.

3.3 | N-ERD airway EVs trigger impaired mediator responses in macrophages, while eliciting pro-inflammatory cytokines in airway epithelial cells

As eicosanoids and chemokines are key mediators in N-ERD, we assessed whether sputum EVs from N-ERD patients may differentially affect the output of these mediators by MDM. In line with the impaired induction of the COX pathway by N-ERD EVs (Figure 2), EVs from healthy individuals, but not from N-ERD patients elicited a significant production of prostanoids (particularly TXB_2, $PGF_{2\alpha}$, PGD_2, and PGE_2) by MDM (Figure 3A). Furthermore, healthy sputum EVs triggered the production of multiple cytokines, in particular TNFaα, IL-6, IL-18, and IL-27, which are involved in the negative regulation of type 2 immune responses and macrophage M2 polarization (Figure 3B).[31–33] In contrast, N-ERD sputum EVs failed to elicit the production of type 2 suppressive cytokines in MDM, suggesting that immunoregulatory roles of EVs are defective in N-ERD (Figure 3B). In line with this result, CCL17, involved in type 2 inflammation,[34] was upregulated by both healthy and N-ERD-derived sputum EVs (Figure 3B). While the levels of most cytokines were much lower in sputum EVs as compared to MDM, EVs contained high amounts of IL-12 family cytokines (IL-12 and IL-27), which may contribute to different immunostimulatory capacities of N-ERD EVs (Table S3). As BALF-derived EVs isolated from asthmatic patients have been shown to alter mediator responses of airway epithelial cells,[10] we quantified the cytokine production of normal human bronchial epithelial cells (NHBEs) in response to sputum EVs from healthy individuals or N-ERD patients. NHBEs exposed to sputum EVs from healthy donors showed no significant induction of epithelial cytokines (Figure 3C). In contrast, sputum EVs from N-ERD patients elicited the production of GM-CSF and IL-8, two cytokines involved in granulocyte recruitment and survival. This suggests that EVs from the airways of N-ERD patients display a reduced potential to trigger the production of type 2 suppressive cytokines and prostanoids in macrophages, while increasing epithelial cytokines that stimulate granulocyte recruitment. Thus, N-ERD airway EVs affect multiple cellular events that favor type 2 inflammation, while impairing type 1 mediated host defense.

3.4 | N-ERD airway EVs display broadly altered miRNA profiles with upregulation of let-7 family miRNAs

miRNAs have been suggested as the major functional cargo of EVs and EV miRNAs have been implicated in asthma and in altered immunological functions of EVs.[10,16,18] Thus, we performed miRNAseq of airway EVs to define potential differences in the miRNA cargo of N-ERD EVs, which may explain the observed aberrant immunostimulatory capacities. Transcriptomic analysis yielded 19 differentially expressed miRNAs between N-ERD and healthy sputum EVs and 96 between nasal polyp tissue EVs from N-ERD and 119 from CRSwNP patients compared to turbinate tissue EVs from healthy individuals (Table S4). This suggested that the miRNA cargo of airway EVs is broadly altered during type 2 inflammation and affected by the tissue microenvironment in the upper and lower airways. For sputum EVs, miRNAs of the let-7 family (let-7a-5p, let-7f-5p, let-7b-5p, and let-7c-5p) were highly upregulated in N-ERD patients as compared to healthy individuals (Figure 4A,B). miR-125a-5p and let-7c-5p were enriched whereas miR-125b-5p, miR-21-5p, miR-200a-3p, miR-30a-5p, miR-155-5p, and miR-3168 were downregulated in N-ERD sputum EVs (Figure 4A,B). Intriguingly, N-ERD sputum EVs showed a more diverse miRNA cargo compared to healthy sputum EVs with only three unique miRNAs in healthy EVs (Figure 4C).

In line with the miRNA profiling of N-ERD sputum EVs, nasal tissue EVs of N-ERD and CRSwNP patients showed high levels of miR-125a-3p and let-7f-5p (Figure 4D,E, Figure S1B,C), prompting us to focus on let-7 miRNAs, implicated in type 2 inflammation and regulation of macrophage functions.[35–37] Furthermore, miR-1246, miR-182-5p, miR-223-5p, and miR-9-5p were upregulated in N-ERD and CRSwNP nasal tissue-derived EVs whereas miR-143-3p and miR-145-3p were downregulated predominantly in CRSwNP nasal polyp EVs (Figure 4D,E, Figure S1B,C). Comparing the miRNA cargo of EVs isolated from healthy nasal tissue, N-ERD or CRSwNP nasal polyp tissue, CRSwNP EVs displayed 140 and N-ERD EVs 45 unique miRNAs compared to healthy nasal EVs (Figure 4F, Figure S1D). This suggests that EVs of the upper and lower airways from N-ERD and CRSwNP patients carry an aberrant miRNA cargo compared to healthy controls. Nasal polyp tissue EVs from N-ERD and CRSwNP patients showed a similar pattern of highly upregulated miRNAs, but an overall distinct miRNA expression pattern with unique miRNAs (e.g., miR-651-5p for N-ERD and miR-449a for CRSwNP) for both endotypes. Thus, EV miRNA profiles in NSAID tolerant and intolerant CRSwNP indicate overlapping pathomechanisms and inflammatory

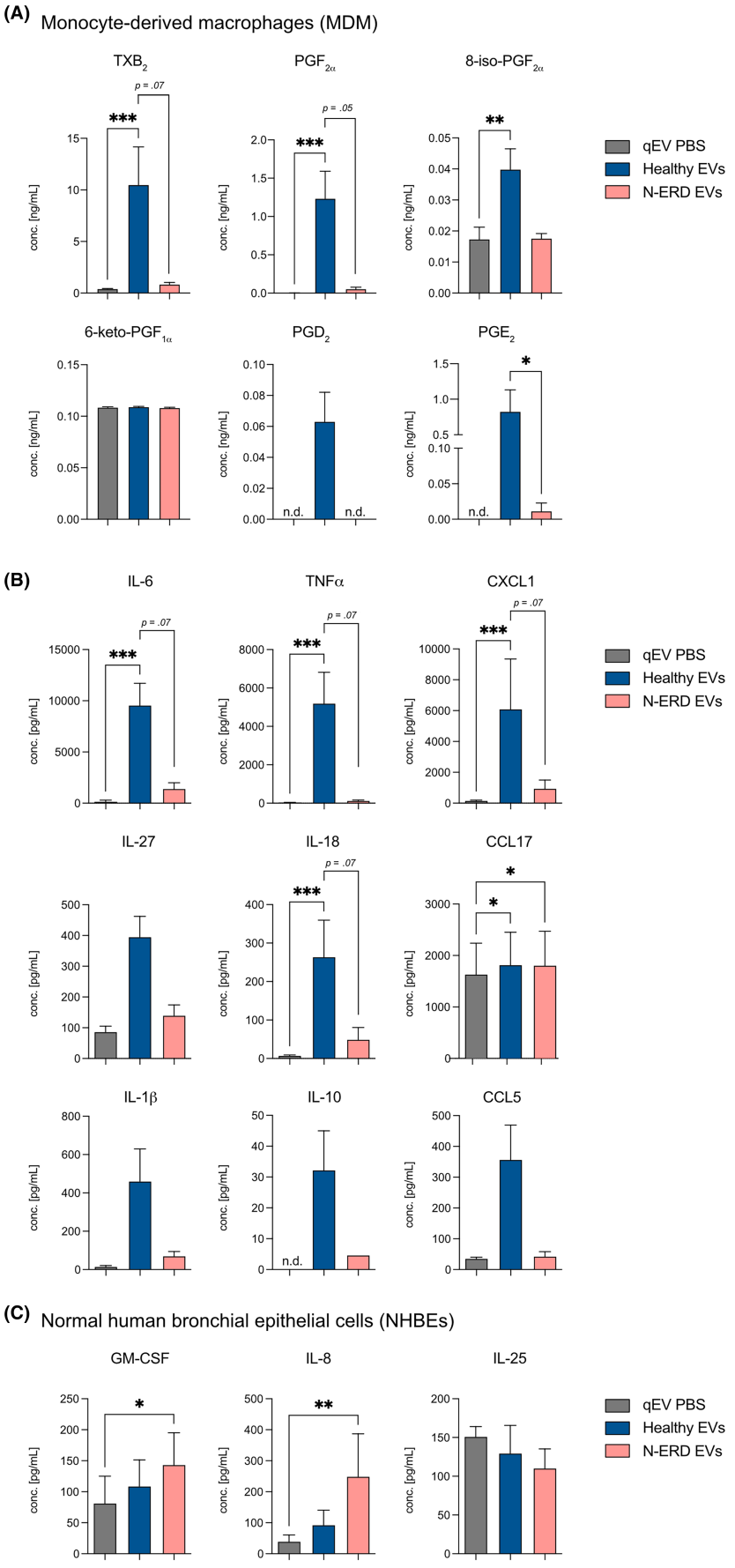


FIGURE 3 N-ERD airway EVs impair cytokine and prostanoid responses in macrophages and bronchial epithelial cells. Mediator production of MDM (A, B) or NHBEs (C) stimulated with sputum EVs from healthy individuals or N-ERD patients: Levels of eicosanoids (LC–MS/MS) (A) and cytokines (multiplex cytokine analysis) for MDM SN ($n=8$) (B) or NHBE SN ($n=4$–5) (C) stimulated with sputum EVs from N-ERD patients or healthy controls or PBS (mock isolated qEV PBS) for 24 h. Data are pooled from two individual experiments and are presented as mean + SEM. Statistical significance was determined by Friedman test with Dunn's multiple comparison test (A–C); $^{*}p<.05$; $^{**}p<.01$; $^{***}p<.001$; n.d. not detected.

signatures, while supporting the potential of distinct EV miRNAs as biomarkers.

3.5 | miRNAs identified in sputum EVs and MDM from N-ERD or healthy subjects differentially regulate M2 macrophage polarization and cytokine responses

As MDM from N-ERD patients displayed an inflammatory metabolic and epigenetic reprogramming,[5] we analyzed miRNA profiles of MDM from healthy controls, N-ERD and CRSwNP patients by small RNA sequencing to assess endogenous miRNA expression of MDM. Fifty-nine or 115 miRNAs were differentially expressed between N-ERD or CRSwNP MDM compared to healthy MDM, respectively (Figure 5A,B, Figure S1E,F, Table S5). miRNAs of patient-derived MDM (N-ERD and CRSwNP) showed a high overlap characterized by upregulation of miRNAs associated with M2 macrophage polarization (miR-125a-3p[38] and miR-125a-5p[39]) and impaired antibacterial immunity (miR-99b[40]) indicating common characteristics of the underlying pathology and immune dysregulation.

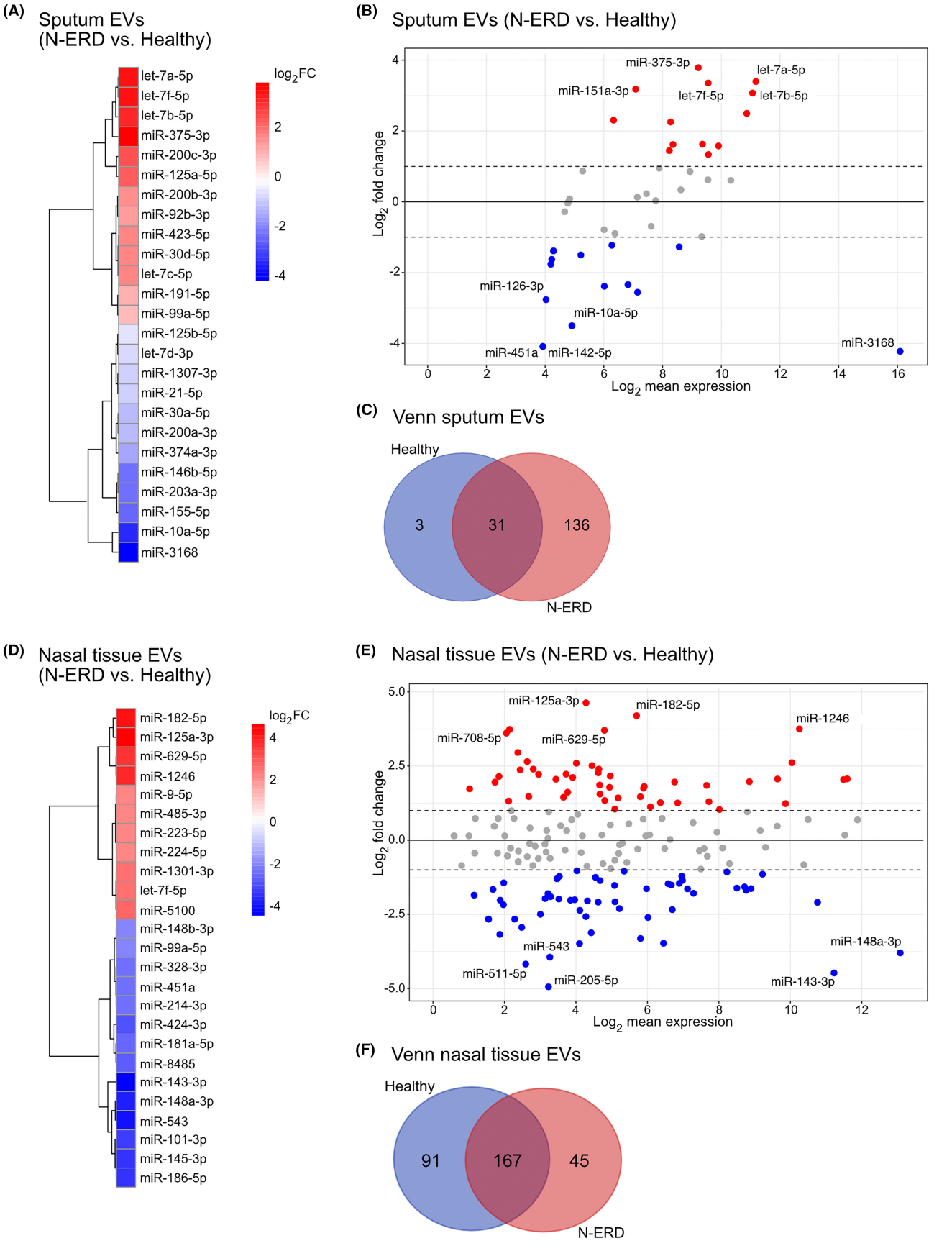
To evaluate the role of the identified miRNA cargo from N-ERD and healthy sputum EVs, we transfected the top differentially regulated miRNAs into MDM and analyzed cytokine and chemokine responses as well as macrophage polarization markers. To control for potential effects of glucocorticoids (GC) on major differentially expressed miRNAs, we analyzed let-7a, let-7f, and miR-155 expression in MDM and NHBEs treated with fluticasone propionate (FP). Short-term (24 h) treatment with FP did not affect let-7a, let-7f, and miR-155 expression in MDM, while FP treatment during the differentiation period (7 days) tended to induce let-7a and let-7f (Figure S3A). As long-term FP treatment during macrophage differentiation impaired cell viability, we additionally studied effects in fully differentiated MDM. However, none of the differentially expressed miRNAs was significantly affected by prolonged FP exposure both in MDM or in NHBEs, (Figure S3B,C), suggesting that GC treatment is not the major driver of miRNA changes in major source cells of airway EVs.

For transfection experiments, we used miR-155-5p and miR-3168 as miRNAs associated with healthy and let-7a-5p as a miRNA cargo of N-ERD sputum EVs. MDM were stimulated with LPS or IL-4 to trigger inflammatory M1 or M2 macrophage activation, respectively. In IL-4-stimulated MDM, let-7a-5p reduced the expression of anti-inflammatory *IL10* compared to control miRNA. Type 2 activating markers (*MRC1* and *TGM2*) were reduced, while *TNF* and *IDO1* were increased by healthy-associated miR-155-5p compared to N-ERD associated let-7a-5p (Figure 5C). Similar results were observed in LPS-treated MDM (Figure 5D) except for *IL6* which was reduced in let-7a-5p-compared to miR-155-5p-treated macrophages. Compared to let-7a-5p and miR-155-5p, miR-3168 had minor effects on EV-modulated genes, showing a weak downregulation of *IL10* and induction of *IDO1* both in M1 and M2 macrophages (Figure S3D,E). As MDM express let-7a-5p endogenously (Figure S3F), we transfected MDM with a let-7 inhibitor targeting let-7a-5p, let-7f-5p and miR-98-5p, all upregulated in N-ERD sputum EVs. Let-7 inhibition resulted in enhanced *IL10* and *TNF*; and reduced *MRC1* expression in IL-4- as well as LPS-stimulated macrophages compared to a control inhibitor (Figure 5E,F). Inhibition of macrophage intrinsic let-7 family miRNAs tended to increase *IL6* and *IDO1* in IL-4-treated MDM, suggesting that N-ERD-associated let-7 miRNAs may promote a shift from M1 towards M2 activation. Both inhibitors (let-7 and control) resulted in low cytotoxicity, which is unlikely to explain differential effects on macrophage activation (Figure S3G). Inflammatory and immunoregulatory tendencies of let-7 inhibition on gene regulation were confirmed under M0 conditions without IL-4 or LPS stimulations (Figure S3H). Thus, immunoregulatory miRNAs contained in healthy airway EVs reduce M2 macrophage polarization and promote cytokine responses contrary to N-ERD associated let-7 family miRNAs, which may contribute to aberrant macrophage effector functions in N-ERD patients.[5]

3.6 | N-ERD sputum EVs prevent immunoregulatory effects of let-7 inhibition

As let-7 is endogenously expressed and let-7 inhibition had profound effects on the transcription of inflammatory cytokines and type 2 suppressive factors in MDM (Figure 5E,F, Figure S3H), we assessed the functional roles of N-ERD sputum EVs during let-7 inhibition (Figure 6A). In line with the findings shown in Figure 3B, exposure to N-ERD EVs alone did not alter the expression or release of *TNF* or *IL6* as well as further immune regulatory genes associated with M2 polarization compared to mock (qEV PBS) stimulated MDM (Figure 6B–D). Interestingly, when let-7 inhibitor was co-transfected with N-ERD sputum EVs, N-ERD EVs prevented the effect of let-7 inhibition resulting in blunted expression and protein levels of type 2 suppressive factors (*TNFAIP3*, *IL6*, and *IDO1*) (Figure 6B,D). In contrast, expression of the M2 marker *TGM2* was partially restored

FIGURE 4 N-ERD airway EVs display broadly altered miRNA profiles with upregulation of let-7 family miRNAs. (A–C) Differential miRNA content (small RNAseq) in sputum EVs between N-ERD patients (pooled, $n = 5$) and healthy individuals (pooled, $n = 10$); (A) Heatmap of top 25 differentially expressed miRNAs, (B) MA plot of detected miRNAs, upregulated miRNAs in red ($\log_2$ fold change >1) with top five labeled, downregulated miRNAs in blue ($\log_2$ fold change <1) with top five labeled, respectively; (C) Venn diagram of distinct miRNA profiles between N-ERD and healthy airway EVs; (D–F) Differentially expressed miRNAs in EVs of nasal tissue from healthy individuals ($n = 1$) and N-ERD patients (pooled, $n = 4$); (D) Heatmap of top 25 differentially expressed miRNAs; (E) MA plot of detected miRNAs in EVs of nasal tissue between N-ERD versus Healthy, upregulated miRNAs in red ($\log_2$ fold change >1) with top five labeled, downregulated miRNAs in blue ($\log_2$ fold change <1) with top five labeled, respectively. (F) Venn diagram of distinct miRNA profiles.


(A)
Sputum EVs
(N-ERD vs. Healthy)
log2FC
2
0
-2
-4
let-7a-5p
let-7f-5p
let-7b-5p
miR-375-3p
miR-200c-3p
miR-125a-5p
miR-200b-3p
miR-92b-3p
miR-423-5p
miR-30d-5p
let-7c-5p
miR-191-5p
miR-99a-5p
miR-125b-5p
let-7d-3p
miR-1307-3p
miR-21-5p
miR-30a-5p
miR-200a-3p
miR-374a-3p
miR-146b-5p
miR-203a-3p
miR-155-5p
miR-10a-5p
miR-3168
(B)
Sputum EVs (N-ERD vs. Healthy)
Log2 fold change
Log2 mean expression
miR-375-3p
let-7a-5p
miR-151a-3p
let-7f-5p
let-7b-5p
miR-126-3p
miR-10a-5p
miR-451a
miR-142-5p
miR-3168
(C)
Venn sputum EVs
Healthy
3
31
136
N-ERD
(D)
Nasal tissue EVs
(N-ERD vs. Healthy)
log2FC
4
2
0
-2
-4
miR-182-5p
miR-125a-3p
miR-629-5p
miR-1246
miR-9-5p
miR-485-3p
miR-223-5p
miR-224-5p
miR-1301-3p
let-7f-5p
miR-5100
miR-148b-3p
miR-99a-5p
miR-328-3p
miR-451a
miR-214-3p
miR-424-3p
miR-181a-5p
miR-8485
miR-143-3p
miR-148a-3p
miR-543
miR-101-3p
miR-145-3p
miR-186-5p
(E)
Nasal tissue EVs (N-ERD vs. Healthy)
Log2 fold change
Log2 mean expression
miR-125a-3p
miR-182-5p
miR-1246
miR-708-5p
miR-629-5p
miR-543
miR-148a-3p
miR-511-5p
miR-205-5p
miR-143-3p
(F)
Venn nasal tissue EVs
Healthy
91
167
45
N-ERD

when N-ERD sputum EVs were added to let-7 inhibitor-treated cells (Figure 6C). Thus, airway EVs from N-ERD patients containing high levels of let-7 family miRNAs (including let-7a-5p, let-7f-5p and miR-98-5p) can overcome type 2 suppressive effects of let-7 inhibition. These data confirm key roles of let-7 family members in functional effects of N-ERD EVs (Figure 4A,B) and suggest that airway EVs contribute to immune dysfunction and chronic type 2 inflammation in N-ERD.

4 | DISCUSSION

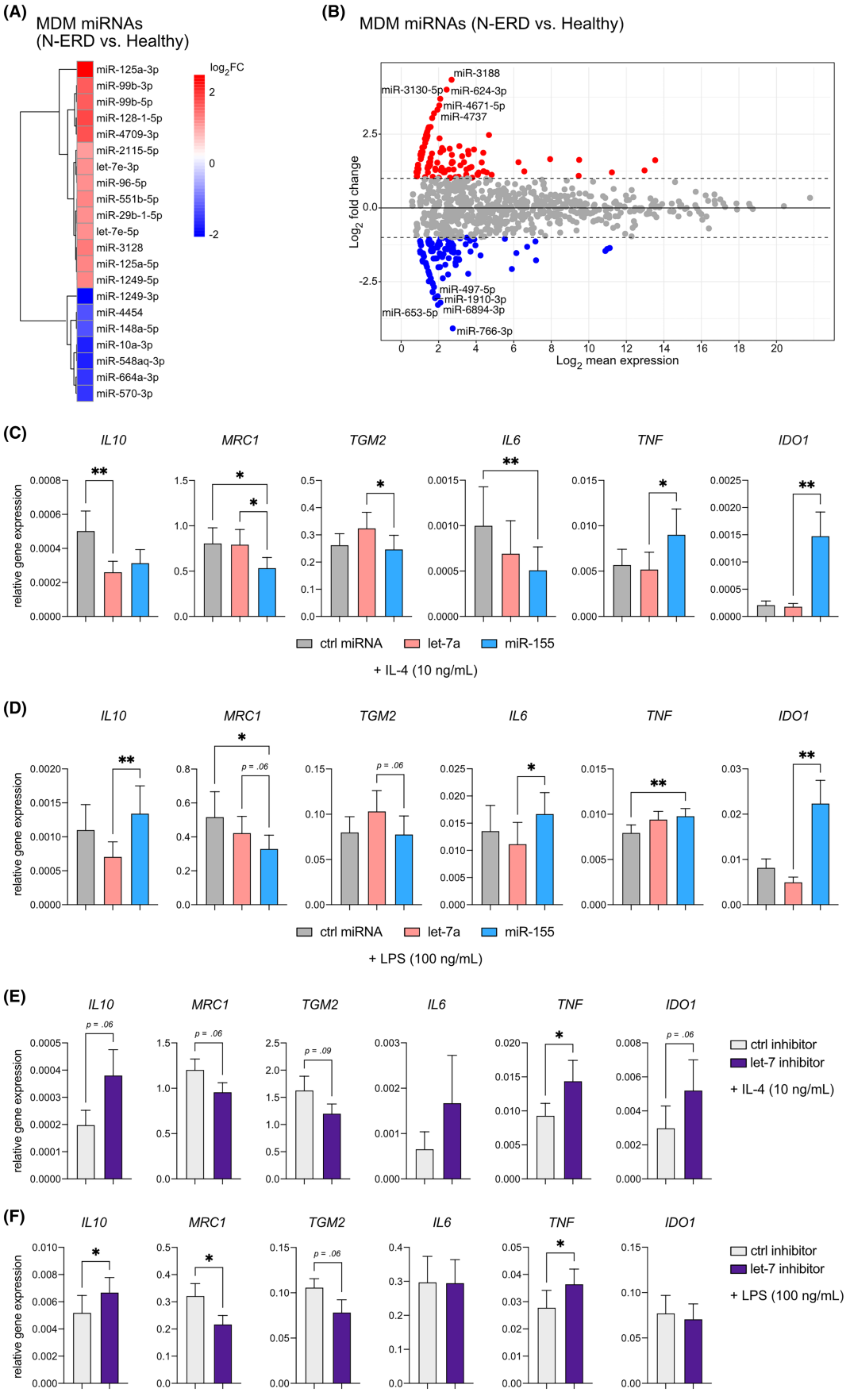
Recent studies have implicated EVs in physiological as well as pathological processes including asthma and chronic rhinosinusitis.[12,41,42] The present study uncovers an altered EV miRNA cargo as a potential pathomechanism in N-ERD and identifies an important role of airway EVs in immune dysregulation and chronic type 2 inflammation.

Intriguingly, healthy airway EVs triggered a profound prostanoid and cytokine response in macrophages, suggesting that EVs can support host defense responses. In contrast, N-ERD EVs showed an impaired capacity to induce factors involved in type 1 immunity (e.g., TNFαa, IL-6) and immune regulation (e.g., PGE_2, IL-10, and IL-18). N-ERD EVs also exhibited a reduced suppression of *FABP4* implicated in neutrophil recruitment[43] and epithelial barrier disruption,[44] suggesting that N-ERD EVs fail to support host defense and barrier integrity. The enhanced induction of MHC class II genes (*HLA-DPB1*, *HLA-DRB1*, *HLA-DOA*, and *HLA-DQB2*) associated with asthma and N-ERD[45–47] further implicates airway EVs in immune dysregulation in N-ERD.

The strong immunostimulatory potential of healthy airway EVs was surprising and may suggest the presence of microbial products within EVs isolated from the airways. Thus, potential differences in the airway microbiome between healthy and N-ERD subjects may at least partially explain their distinct immunological effects. However, in contrast to their reduced stimulatory effects on macrophages, N-ERD airway EVs triggered the release of pro-inflammatory IL-8 and GM-CSF by NHBEs, which was not the case for healthy airway EVs. Thus, the distinct cargo of healthy and N-ERD EVs has specific effects on different cell types implicated in host defense, inflammation, and barrier function in the airways. miRNAs have been implicated in the regulation of gene expression in allergy and asthma[48] and we observed prominent differences in the miRNA content of healthy and patient-derived EVs. We particularly focused on miRNAs of the let-7 family, known to regulate inflammation[35,36,49] and macrophage polarization[37,50] as these were highly abundant in EVs from N-ERD and CRSwNP patients. miR-125a and let-7c, both enriched in N-ERD sputum EVs induce M2 polarization,[37,39] whereas miR-125b, miR-21, miR-155, and miR-200a, all downregulated in N-ERD sputum EVs, are associated with M1 polarization.[51–53] In line with our results, miR-155, miR-3168, and let-7d levels were reduced in patients suffering from asthma or idiopathic pulmonary fibrosis.[16,54,55] Mice deficient for miR-155 displayed increased airway remodeling and Th2 polarization suggesting a homeostatic function,[56] which is defective in N-ERD and may contribute to the type 2 immunopathology. miR-1246, upregulated in nasal tissue-derived patient EVs, was also increased in airway epithelium of asthmatics and its target *POSTN* was shown to regulate asthma and fibrosis progression.[57] This suggests that miRNAs of EVs and MDM from N-ERD and CRSwNP patients promote M2 polarization and aberrant macrophage activation, which may contribute to impaired lung function and nasal polyp growth. Despite our functional assays, supporting a key role for let-7 miRNAs in the reduced immunostimulatory capacities of N-ERD EVs, we cannot exclude a contribution of additional factors, such as cytokines (e.g., IL-12 and IL-27), which we also found to be enriched in healthy sputum EVs. Importantly, treatment with glucocorticoids (GC) might influence the miRNA content in N-ERD- and CRSwNP-derived samples[58–63] and previously reported GC-induced changes in miRNAs (upregulation of let-7 family members and miR-98, downregulation of miR-155 and miR-143) were in line with CRSwNP-associated profiles observed in our study. However, fluticasone propionate did not affect let-7 family miRNA expression in bronchial epithelial cells or macrophages, suggesting that the inflammatory pathology itself rather than GC treatment is the major driver of aberrant miRNA profiles in N-ERD airway EVs. Of note, N-ERD patients undergoing surgery for nasal polyp removal received systemic GC preoperatively, possibly explaining lower ECP levels as compared to patients undergoing sputum sampling.[5] These different treatment regimens may contribute to the lower overlap between healthy and N-ERD EV miRNA profiles in sputum as compared to nasal tissue.

Contrary to our study, multiple studies reported reduced levels of let-7a in bronchial biopsies, serum, and nasal tissue from asthmatics and CRSwNP patients, respectively.[64,65,36] In addition, plasma EVs from asthmatics were shown to display lower levels of let-7a and increased miR-155 expression.[65] Indeed, the tissue milieu and source cells play a pivotal role in miRNA expression profiles and serum or

FIGURE 5 miRNAs identified in sputum EVs and MDM from N-ERD or healthy subjects differentially regulate cytokine responses and M2 macrophage polarization. (A, B) Differentially expressed miRNAs in MDM between healthy individuals ($n=2$) and N-ERD ($n=2$) patients; (A) Heatmap of top 21 differentially expressed miRNAs, (B) MA plot of detected miRNAs between healthy and N-ERD patients, upregulated miRNAs in red (log_2 fold change >1) with top five labeled, downregulated miRNAs in blue (log_2 fold change <1) with top five labeled, respectively; (C–F) Relative gene expression of MDM transfected with control miRNA, miRNAs (let-7a-5p and miR-155-5p) (C, D), or inhibitors (control, let-7 (E, F)) (50 nM) for 48 h stimulated with IL-4 (10 ng/mL) (C, E) or LPS (100 ng/mL) (D, F) ($n=6$). (C–F) Data are pooled from two individual experiments and presented as mean + SEM. Statistical significance was determined by Friedman test with Dunn's multiple comparison test (C, D) or Wilcoxon test (E, F); $^*p<.05$; $^{**}p<.01$.


(A)
MDM miRNAs
(N-ERD vs. Healthy)
log2FC
2
0
-2
miR-125a-3p
miR-99b-3p
miR-99b-5p
miR-128-1-5p
miR-4709-3p
miR-2115-5p
let-7e-3p
miR-96-5p
miR-551b-5p
miR-29b-1-5p
let-7e-5p
miR-3128
miR-125a-5p
miR-1249-5p
miR-1249-3p
miR-4454
miR-148a-5p
miR-10a-3p
miR-548aq-3p
miR-664a-3p
miR-570-3p
(B)
MDM miRNAs (N-ERD vs. Healthy)
miR-3188
miR-3130-5p
miR-624-3p
miR-4671-5p
miR-4737
miR-497-5p
miR-1910-3p
miR-6894-3p
miR-653-5p
miR-766-3p
Log2 fold change
Log2 mean expression
(C)
IL10
MRC1
TGM2
IL6
TNF
IDO1
relative gene expression
ctrl miRNA
let-7a
miR-155
+ IL-4 (10 ng/mL)
(D)
IL10
MRC1
TGM2
IL6
TNF
IDO1
relative gene expression
p = .06
ctrl miRNA
let-7a
miR-155
+ LPS (100 ng/mL)
(E)
IL10
MRC1
TGM2
IL6
TNF
IDO1
relative gene expression
p = .06
p = .09
ctrl inhibitor
let-7 inhibitor
+ IL-4 (10 ng/mL)
(F)
IL10
MRC1
TGM2
IL6
TNF
IDO1
relative gene expression
p = .06
ctrl inhibitor
let-7 inhibitor
+ LPS (100 ng/mL)

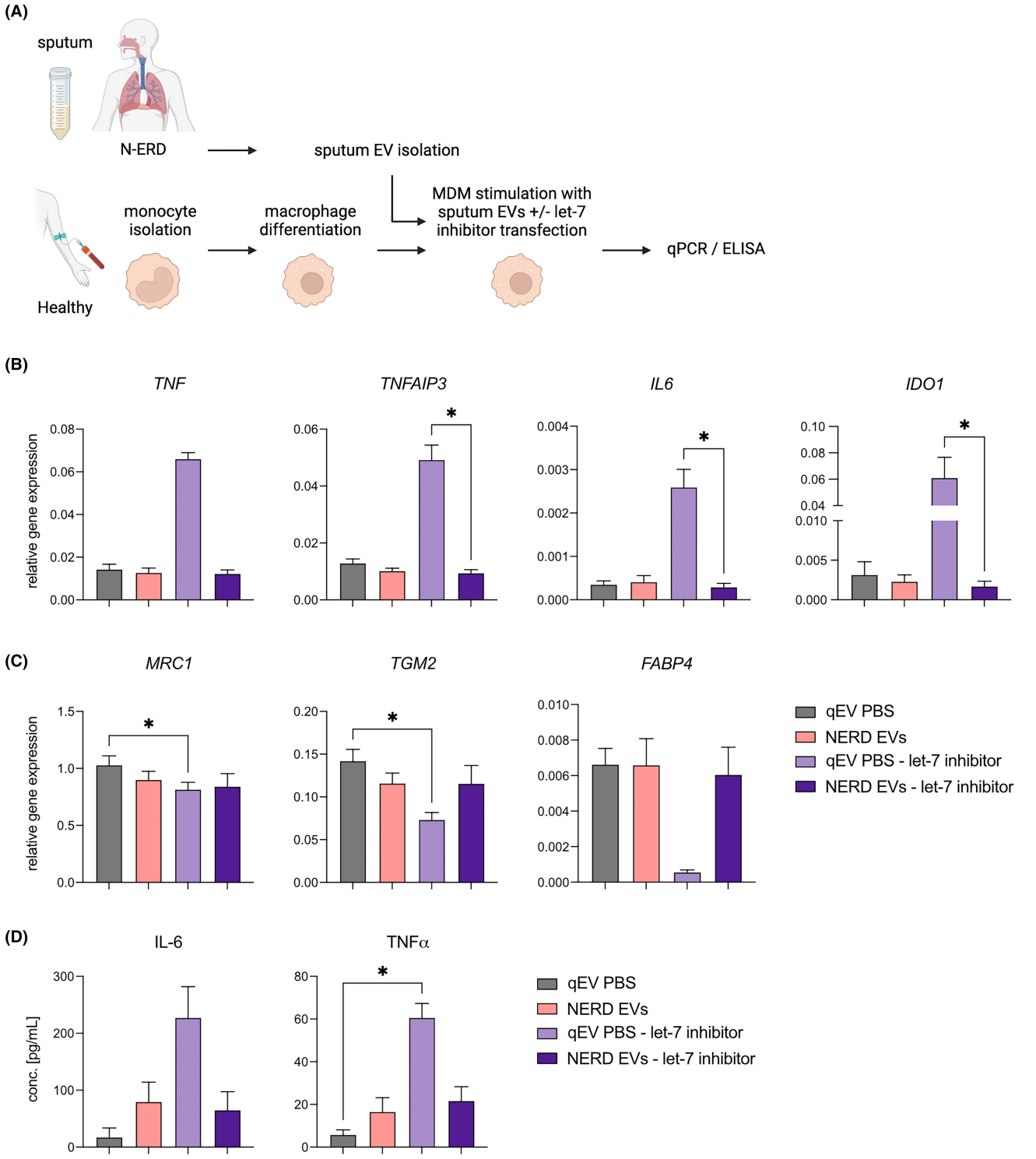


FIGURE 6 N-ERD sputum EVs prevent immunoregulatory effects of let-7 inhibition. (A) Experimental design of MDM transfected with let-7 inhibitor and exposed to N-ERD airway EVs, created with BioRender.com; Gene expression profile (B, C) and protein levels (D) of MDM ($n=3$) +/− transfected with let-7 inhibitor (50 nM) +/− exposed to N-ERD sputum EVs for 48 h. Data are presented as mean + SEM from one experiment. Statistical significance was determined by Friedman test with Dunn's multiple comparison test (B–D); *$p<.05$.

EV miRNA content might not reflect the profiles found in inflamed tissues. Previous studies indicated that EV miRNAs do not resemble host cell miRNA content and that miRNAs negatively correlated between airway EVs and lung tissue which may further explain the reported discrepancies.[66–68] As EVs were isolated from induced sputum, we analyzed the products of a heterogenous mixture of host cells, mostly reflecting the upper airways. Indeed, the miRNA levels of MDM derived from healthy, N-ERD, or CRSwNP patients did not match the miRNA cargo identified within airway EVs, suggesting that MDM are not the main EV/miRNA source in induced sputum. In murine BALF, 80% of EVs are of epithelial origin[69] and epithelial-derived miRNAs and EVs have been implicated in asthma development.[70,19]

However, during allergic airway inflammation, EVs of hematopoietic origin were recruited into the inflamed tissue suggesting a contribution of immune-cell derived EVs to allergic inflammation.[69] Therefore, further research is necessary to elucidate the origin, target cells, and the contribution of airway EVs to the pathogenesis and severity of N-ERD. As obtaining sufficient EV material from individual donors is difficult, EVs from different donors had to be pooled for miRNA sequencing and functional assays. We did not collect sputum from most NSAID tolerant CRSwNP patients as this subgroup had poorly characterized and heterogenous lower airway inflammation. Thus, we decided to focus on N-ERD as a clearly defined CRSwNP endotype with bronchial asthma. A more detailed characterization and comparison of airway EVs based on protein markers was impossible due to the low protein content of the highly pure EV preparations. The low amounts of airway EVs obtained from healthy individuals precluded functional assays with let-7 inhibitors and healthy sputum EVs. We were also unable to purify sufficient amounts of N-ERD EVs for experiments with a control inhibitor, limiting us to the comparison between let-7 inhibitor alone or in the presence of N-ERD EVs. Such limitations could potentially be overcome by recently developed single cell methods, including sc miRNA sequencing,[71] which may be adapted to EVs in the future.

Given the long-term stability of EVs, the impact of GC and current biologics (e.g., dupilumab) on EV/miRNA levels should be investigated to assess the beneficial and detrimental effects of treatment regimens on the susceptibility to infections and exacerbation. Overall, this study implicates airway EV miRNAs in chronic type 2 inflammation and increased susceptibility to respiratory infections in asthma and CRSwNP and suggests that targeting EV miRNAs may correct for aberrant macrophage activation and mediator responses in chronic airway disease.

AUTHOR CONTRIBUTIONS

J.E.v.B. was involved in conceptualization, funding acquisition, and project administration. V.M., B.K., P.H., F.H., S.S., F.D.H., R.S.-M., M.W.P., and U.M.Z. were involved in methodology. F.H., P.H., and S.S. were involved in investigation. V.M., B.K., F.H., and P.H. were involved in visualization. A.M.C., U.B., and U.M.Z. were involved in patient recruiting. A.M.C. and U.B. were involved in EC approval. J.E.v.B. and C.S.-W. were involved in supervision. J.E.v.B. and F.H. were involved in writing—original draft.

ACKNOWLEDGMENTS

We thank all patients and healthy individuals for participating in this study. Additionally, we would like to acknowledge the help of Mark Haid, Jerzy Adamski, Antonie Lechner, Marta de Los Reyes Jiménez, and the Helmholtz Center Munich Genomics core facility. Mark Haid and Jerzy Adamski for support with LC-MS/MS analysis. Open Access funding enabled and organized by Projekt DEAL.

FUNDING INFORMATION

This study was supported by the Else Kröner-Fresenius-Stiftung (grant 2015_A195), by grants of the German Research Foundation (FOR2599, ES 471/3-2, SPP2306, ES 471/7-1, and ES 471/6-1) and a Helmholtz Young Investigator grant by the Helmholtz Initiative and Networking fund VH-NG-1331 to J.E.v.B.

CONFLICT OF INTEREST STATEMENT

All authors have no conflict of interest in relation to this work.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Hartung F, Haimerl P, Schindela S, et al. Extracellular vesicle miRNAs drive aberrant macrophage responses in NSAID-exacerbated respiratory disease. *Allergy*. 2024;79:1893-1907. doi:10.1111/all.16117