IMMUNOMODULATORY ROLES OF EXTRACELLULAR VESICLES IN AUTOIMMUNE DISEASES

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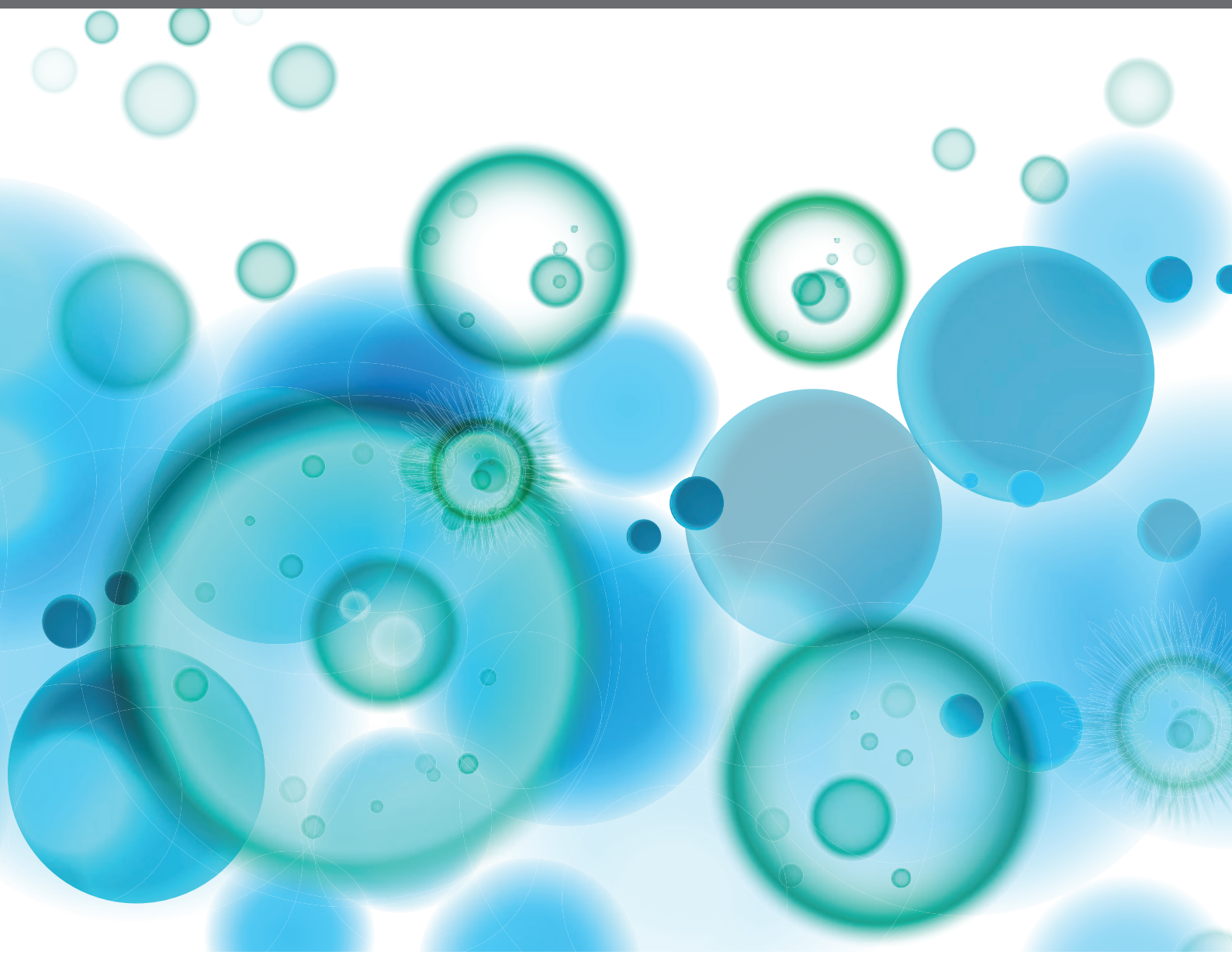

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IMMUNOMODULATORY ROLES OF EXTRACELLULAR VESICLES IN AUTOIMMUNE DISEASES

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EDITORIAL
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Editorial: Immunomodulatory Roles of Extracellular Vesicles in Autoimmune Diseases

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Keywords: extracellular vesicles, autoimmune diseases, diagnosis, immunomodulatory, pathogenesis, therapeutics

Editorial on the Research Topic

Immunomodulatory Roles of Extracellular Vesicles in Autoimmune Diseases

Autoimmune diseases reflect a breakdown in self-tolerance that results from defects in our complex immune system. The mechanisms are multifactorial and include a combination of cellular, genetic, epigenetic, and molecular components that result in phenotypic inflammatory responses in a variety of tissues and organs. Extracellular vesicles (EVs) have been shown to play a role in immunomodulation and pathogenesis of autoimmune diseases. This Research Topic provides a few reviews and manuscripts highlighting the immunomodulation and therapeutic potential of EVs in a variety of autoimmune diseases including rheumatoid arthritis (RA), Sjögren's syndrome (SS), bullous pemphigoid (BP), type 1 diabetes mellitus (T1DM), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), and antiphospholipid-associated diseases (APS).

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IMMUNOMODULATORY EFFECTS OF MESENCHYMAL STEM CELLS AND MESENCHYMAL STEM CELL-DERIVED EXTRACELLULAR VESICLES IN RHEUMATOID ARTHRITIS

RA is a chronic autoimmune disease affecting joints *via* pain, inflammation, loss of mobility, and eventually the erosion of joints, with no effective treatment to date. As reviewed in this Research Topic, extracellular vesicles derived from mesenchymal stem cells (MSCs) are more stable, less toxic, and more effective at transferring nucleic acids, proteins, and lipids from parent to recipient cells. MSC-EVs are multipotent progenitor cells with immunomodulatory properties that can be easily obtained and expanded rapidly, as well as a potential therapeutic strategy for RA (Liu et al.).

RECENT ADVANCES IN THE USE OF EXOSOMES IN SJÖGREN'S SYNDROME

SS is a chronic multi-organ autoimmune disorder primarily effecting the exocrine glands. As reviewed in this Research Topic, ongoing extracellular vesicle studies in SS have been limited to tears and saliva, neglecting the investigation of novel biomarkers and potential therapeutic effects of EVs in SS from effected tissues and organs (Huang et al.).

ASSESSMENT OF THE CHARACTERISTICS AND ASSOCIATED FACTORS OF INFECTIOUS COMPLICATIONS IN BULLOUS PEMPHIGOID

BP is an autoimmune disorder of the skin characterized by blistering, urticarial lesions, and itching. In this retrospective study, Chen et al. demonstrated that inpatient BP patients are at risk of infectious complications, leading to comorbidities, due to higher doses of corticosteroids. They compared the risk factors of infection of inpatients and outpatients to develop preventative and treatment strategies.

COMMENTARY: PROINFLAMMATORY ROLE OF BLISTER FLUID-DERIVED EXOSOMES IN BULLOUS PEMPHIGOID

Liu and Li proposed the potential use of EVs for the understanding the proinflammatory roles of BP for diagnostics. As shown in studies, concentrations of cytokines are elevated in BP, and it is speculated that EVs can transport the pathogenic autoantibodies associated with BP which can then be released and stimulate a favorable immune response.

MOLECULAR AND FUNCTIONAL DIVERSITY OF DISTINCT SUBPOPULATIONS OF THE STRESSED INSULIN-SECRETING CELL'S VESICULOME

In this study, Giri et al. investigated the changes in the relative composition of the vesiculome as well as the partition of the candidate autoantigen insulin and immunostimulatory miRNA sequences inside apoptotic bodies, microvesicles, and exosome subpopulations derived from equal amounts of healthy and stressed beta cells and their impact on innate immune responses. They identified that beta small extracellular vesicles (sEVs) have been shown to drive innate and adaptive pro-diabetogenic immune responses, with a limitation in the molecular and functional diversity of EVs in the beta cell's secretome, which necessitates further exploration.

EXTRACELLULAR VESICLES IN RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOSUS: FUNCTIONS AND APPLICATIONS

In this review, Zhang et al. reviewed recent studies examining the roles of EVs in RA and SLE, both chronic autoimmune diseases but SLE affects multiple organs, in understanding their pathogenesis, diagnosis, and therapeutic potentials.

NEUTROPHIL EXTRACELLULAR TRAPS TIED TO RHEUMATOID ARTHRITIS: POINTS TO PONDER

Neutrophils play a central role in our immune defense system with pathogen clearance, immune regulation, and disease pathology. Song et al. describe the role of neutrophil extracellular traps (NETs) in detail, as novel therapeutic targets for RA.

EXTRACELLULAR MIR-574-5P INDUCES OSTEOCLAST DIFFERENTIATION *VIA* TLR 7/8 IN RHEUMATOID ARTHRITIS

Hegewald et al. detail the roles of sEVs carrying microRNAs (miRs) in RA. The sEVs from synovial fluid promote osteoclast differentiation, attributed to high levels of extracellular miR-574-5p. They continue to demonstrate that enhanced osteoclast maturation is mediated by toll-like receptor (TLR) 7/8 signaling due to the mechanism of miR-574-5p binding. This is a novel finding of the role of miR-574-5p which may provide a therapeutic approach to protect osteoclast-mediated bone destruction in RA.

GRANULOCYTIC MYELOID-DERIVED SUPPRESSOR CELL EXOSOMAL PROSTAGLANDIN E2 AMELIORATES COLLAGEN-INDUCED ARTHRITIS BY ENHANCING IL-10^{+} B CELLS

In this comprehensive study, Wu et al. identified granulocytic-myeloid suppressor cell (G-MDSC)-derived EVs as a potential mediator in the treatment of mice with collagen-induced arthritis (CIA). The initial finding demonstrated lower arthritis index values and decreased inflammatory cell infiltration, indicating an alteration of the humoral environment by mediating high levels of prostaglandin E2 (PGE2), by production of IL-10^{+} B cells.

EMERGING ROLES OF EXOSOMES IN T1DM

T1DM is caused by an immune-mediated destruction of pancreatic beta cells. Pang et al. detail a comprehensive review on the understanding of how exosomes can 1) enable the underlying pathogenic mechanisms of T1DM, 2) provide novel biomarkers for T1DM diagnosis, and 3) lead to the development of new TIDM therapeutic strategies.

OLFACTORY ECTO-MESENCHYMAL STEM CELL-DERIVED EXOSOMES AMELIORATE EXPERIMENTAL COLITIS *VIA* MODULATING TH1/TH17 AND TREG CELL RESPONSES

In this extensive study, Tian et al. have identified the immunoregulatory property of exosomes derived from olfactory ecto-mesenchymal stem cells (OE-MSCs) and their immunomodulation capacity to ameliorate disease severity in IBD mice, primarily by regulating Th-cell immune responses. Their study suggests OE-MSC exosomes are a potential novel cell-free therapy for targeting inflammatory diseases.

EXOSOME-CONTAINED APOH ASSOCIATED WITH ANTIPHOSPHOLIPID SYNDROME

APS is a systemic autoimmune disorder in which the body's immune system makes antibodies that attack phospholipids which can lead to thrombosis and/or pregnancy complications. Tan et al. conducted human and mouse studies to demonstrate that APS exosomes are a key factor in the pathogenesis of APS and that apolipoprotein H (APOH) is a protein that impairs vascular biological function. They concluded APS and APOH exosomes impair vascular development (pathogenesis) and lead to pregnancy complications, providing new targets for therapeutic intervention.

EXTRACELLULAR VESICLES SECRETED BY MESENCHYMAL STROMAL CELLS EXERT OPPOSITE EFFECTS TO THEIR CELLS OF ORIGIN IN MURINE SODIUM DEXTRAN SULFATE-INDUCED COLITIS

Tolomeo et al. compared the effects of MSCs and of MSC-EV administration in mice with colitis induced by dextran sulfate sodium (DSS). They reported naïve MSCs and induced MSC administration resulted in poor clinical and histological outcomes, with pro-inflammatory polarization of intestinal macrophages. However, when the mice were treated with induced EVs, there was decreased intestinal fibrosis and angiogenesis and a striking increase in intestinal expression of Mucin 5ac, suggesting improved epithelial function. EVs demonstrated a beneficial effect, more predictable behavior, a safer therapeutic profile, and efficacy with respect to their cells of origin.

CONCLUSIONS

This Research Topic has provided examples in which EVs have displayed immunomodulatory roles in autoimmune diseases. In addition, it highlighted and suggested the necessity for future discovery of new targets for therapeutic interventions which will aid in the diagnosis, understanding the mechanisms, and outcomes for a variety of autoimmune diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of Interest: WK is employed by Predicine.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Assessment of the Characteristics and Associated Factors of Infectious Complications in Bullous Pemphigoid

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Objectives: The clinical outcome of bullous pemphigoid appears worse in patients with infectious complications, and assessment of the prevalence and risk factors of infectious complications could be necessary to plan preventative strategies and to instruct the treatment plans. We sought to determine the risk factors of infection and compare associated factors in inpatients and outpatients with different system infections.

Design: This is a single-centered retrospective study on the medical records of 252 patients from 2010 to 2018 at the dermatology department, Peking Union Medical College. Medical profiles of medical history, diagnosis, infectious complications, and treatment plans were analyzed. The associated factors were compared between the subgroups, including inpatients and outpatients, different body sites of infection.

Results: Of the total 252 patients with bullous pemphigoid (BP), 81 patients (81/252, 32.1%) had infectious complications. Forty-eight patients died from pulmonary infections (11/48, 22.9%), cardiovascular diseases (6/48, 12.5%), and other diseases. Infections were most frequently found in skin/mucosa (44/252, 17.5%), respiratory system (32/252, 12.7%), and blood (10/252, 4.0%). On multivariate analysis, risk factors of infections in BP were maximal control dose of corticosteroids (OR 2.539, 95% CI 1.456–4.430, $p = 0.001$), low serum albumin level (OR 2.557, 95% CI 1.283, 5.092, $p = 0.007$), hospitalization (OR 4.025, 95% CI 2.289, 7.079, $p < 0.001$), comorbidities including respiratory disease (OR 4.060, 95% CI, 1.861, 8.858, $p < 0.001$), eye disease (OR 4.431, 95% CI 1.864, 10.532, $p < 0.001$), and diabetes (OR 2.667, 95% CI 1.437, 4.949, $p = 0.002$). The rate of infection was significantly higher in inpatients compared to that in outpatients (54.0 vs. 20.6%, $p < 0.001$), with diverse risk factors. Mucocutaneous infections were associated with a maximal control dose of corticosteroid and other dermatoses. Respiratory infections were related to respiratory disease and old age, and hematologic infection was associated with low serum hemoglobin levels and mucosal involvement of BP. Both of them were associated with mucosal involvement of BP and high titer anti-BP180 antibody.

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Conclusions: Infectious complications of bullous pemphigoid are common and are associated with mucosal involvement of BP, more comorbidities, the higher dose of corticosteroids, and the lower level of serum albumin.

Keywords: pemphigoid, infections, outpatients, corticosteroids, serum albumin

INTRODUCTION

Bullous pemphigoid (BP) is an autoimmune bullous skin disorder commonly identified in the elderly population. It is an autoantibody-induced cutaneous inflammatory disease against BP180 or BP230 at the dermal-epidermal junctions (1, 2). The annual incidence rate of BP has been increasing steadily in the elderly and general populations (3), and the BP patients were reported to have a significantly increased risk of death compared to the control subjects (4–7). Conditions such as older age, poor general condition, dementia, comorbidities, and high-dose corticosteroids have been reported to be the predisposing factors for death (8–11). Prior studies have indicated that infection is the leading cause of death in BP patients. In a published series, almost all BP patients treated with corticosteroids had at least one localized or systemic infection during the follow-up period, and 43% of those patients experienced systemic infections that require hospitalization or lead to death (12). Moreover, approximately one-third of BP patients developed localized skin infections, with 10% of fatal necrotizing fasciitis at 1 year after treatment with topical corticosteroids (13), supporting that infections occurring after the onset of BP tended to worsen the clinical outcomes (14–16). Consequently, the evaluation of the prevalence and risk factors of infectious complications could be indispensable for better preventative strategies and treatment plans with corticosteroids or other immunosuppressive drugs for these patients (14).

Given the importance of infections in BP prognosis and management, only a few studies have been published on the risk factors of infection (12, 17, 18). Those studies were limited with a small number of patients and emphasized the severe infections contributing to hospitalization or mortality. Moreover, previous researches focused mainly on inpatients but less on outpatients. In the current work, we enrolled a relatively large cohort of 252 BP patients, including 87 inpatients and 165 outpatients. The goal of our study is to: (1) retrospectively analyze and compare the clinical characteristics of infected BP patients in inpatients and outpatients, (2) determine the risk factors of infection by analyzing the comorbidities, blood test results and treatment choices, and (3) compare the associated factors in patients with infections at different body sites. The results from our BP patients' medical records were analyzed, discussed, and compared to those from previous studies.

MATERIALS AND METHODS

Patients

Following the principles of the Declaration of Helsinki, this study was approved by the Ethical Committee of Peking Union Medical College Hospital (S-K965). We identified patients with bullous pemphigoid between 2010 and 2018 at the Department of Dermatology, PUMCH. The diagnosis of bullous pemphigoid (BP) was based on the S2k guideline for the diagnosis of bullous pemphigoid (19). Patients with one of the three constellations below were included in our study: (1) compatible clinical picture, and either corresponding histopathology or positive direct IF microscopy, and either epidermal binding of IgG in indirect IF microscopy (on split skin or monkey esophagus) or reactivity with BP180 antibody; (2) clinical picture with tense blisters, and epidermal binding of IgG in indirect IF microscopy, and either corresponding histopathology or reactivity with BP180 antibody; (3) clinical picture with tense blisters, and reactivity with BP180 antibody higher than 27 U/ml. Patients without a record of medical history, basic serologic tests, and treatment plans for further analysis were ruled out. Results of the BP230 antibody were obtained only in one patient, so it was not considered as a part of diagnostic criteria. We included and followed up both outpatients and inpatients with BP until Dec 2019.

Data Collection

Patients' files were reviewed to collect baseline information (i.e., gender, age, and birthplace), demographic characteristics and skin lesion distribution, results of clinical tests on the diagnosis of diseases before infectious complications, the history of past illness, comorbidities, and the treatment plans. Infections that did not result in clinical symptoms were not included because of the low number and thus were less likely to affect clinical prognosis in patients with BP. The information for disease severity was not available for analysis, but the skin lesions were documented in detail in the medical records.

Statistical Analysis

Data were investigated first for all inpatients and outpatients diagnosed with BP and were further analyzed for all patients with infectious complications. Comparisons of the associated factors between subgroups (different sites of infection) were conducted. Descriptive statistics were applied to report the baseline characteristics, demographics, test results, past medical history, comorbidities, and treatment variables (genres of immunosuppressant, maximum control dose of corticosteroid). Student's *t*-test or Mann-Whitney U test was used in continuous outcomes with a normal distribution or not. Categorical data were first analyzed with the Chi-square test or Fisher's exact test and then reported as the relative risk with 95% confidence intervals and *P* values. We made a multivariate analysis, using binary outcome and incorporating the factors found significant by univariate analysis and those deemed clinically significant. Statistical

analyses were performed using software (SPSS, Version 25, IBM Corp., Armonk, NY; RStudio, Version 1.2.1335). All tests were two-tailed, and $p < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics

We searched for the hospital information system and found that 383 patients were initially diagnosed with BP from 2010 to 2018. One hundred two of them were ruled out because of doubtful diagnosis, and 29 of them were excluded due to a lack of data for further analysis. Eventually, a total number of 252 patients were included, and 81 of them were diagnosed with infectious complications after BP onset. Among them, 48 patients died due to pulmonary infections (11/48, 22.9%), cardiovascular diseases (6/48, 12.5%), cerebral infarction (5/48, 10.4%), BP relapse (4/48, 8.3%), cancer (3/48, 6.3%), digestive diseases (2/48, 4.2%), and other unknown reasons (17/48, 35.4%). The patients were followed up for an average of 2.9 ± 0.2 years from the beginning of diagnosis.

The female to male ratio was 1.2:1, with an average age of 67.2 years old at BP onset. The median interval from the onset of BP to diagnosis was 9.1 months (**Table 1**). 67.1% of the patients only had skin involvement, and 24.2% of the patients had both skin and mucosal involvement of BP. The oral mucosa was the most frequently affected mucosa in BP. All patients were followed up in our outpatient clinic. 34.5% were admitted as inpatients for an average of 23.2 days in the hospital. 74.6% of the patients were treated with oral or intravenous corticosteroids, 52.0% with immunosuppressants, and 3.6% with IVIG. One hundred fifteen patients were treated with only one, and 16 patients with two or three immunosuppressants (**Supplementary Table 1**). Additional therapy adjuvants include minocycline, nicotinamide, and topical corticosteroids.

TABLE 1 | Demographic and clinical features of all BP patients.

Features	*N* = 252
Age/year (average, median)	67.2 ± 1.0, 69.1
Gender	
Male (*n*, %)	137 (54.4)
Female (*n*, %)	115 (45.6)
Duration before diagnosis of BP/month	9.1 ± 1.2, 3.0
Follow-up period/year	2.9 ± 0.2, 2.5
Distribution	
Skin only (*n*, %)	169 (67.1)
Skin and mucosa (*n*, %)	61 (24.2)
Mucosa only (*n*, %)	5 (2.0)
Hospitalization	
Number of inpatients (*n*, %)	87 (34.5)
Duration/day	23.2 ± 1.9, 19.5
Treatments	
Corticosteroids (*n*, %)	188 (74.6)
Immunosuppressants (*n*, %)	131 (52.0)
IVIG	9 (3.6)
Infections (*n*, %)	81 (32.1)

In total, 252 patients were included. The average ± SD and median values were calculated for age, duration before BP diagnosis, follow-up period, and hospitalization duration, respectively. For each of the other categories, the number (n) and percentage (%) of patients were listed. IVIG, intravenous immunoglobulin.

Infectious Complications

Infectious complications occurred in 81 patients (81/252, 32.1%), of which 50 patients (50/81, 61.7%) had infections within the first year after BP diagnosis. The median duration between BP diagnosis and infection onset was 5 months.

Localized infections are infections in the skin, oral mucosa, and vulva, and systemic infections are infections in the lung (including TB infection, TB reactivation, and pneumonia), upper respiratory tract, urinary tract (UTI), digestive system, blood infection, and central nervous system infection (CNSI) (**Table 2**). Diagnosis of infectious complications was based on clinical manifestations and lab tests such as microbial cultivation, antimicrobial susceptibility tests, DNA, and specific IgG detection. Cutaneous and respiratory infections were the most commonly observed infections. The most common pathogens identified in these patients were the staphylococcus, candida, and cytomegalovirus (CMV), respectively. CNSI was diagnosed in one patient based on clinical manifestations, the biochemical tests of her spinal fluid, and the blood cultures with Listeria monocytogenes.

Risk Factors for Infectious Complications

On univariate analysis, significant risk factors for developing infectious complications include respiratory disease ($p < 0.001$), digestive disease ($p = 0.027$), osteoarthropathy ($p < 0.001$), endocrine and metabolic disease ($p < 0.001$) oculopathy ($p < 0.001$) (**Figure 1**). Laboratory biochemical tests showed that the serum albumin level was lower in the infected group ($p = 0.004$) (**Supplementary Figure 1**). No significant difference was found in other serum lab tests, tumors, neurologic disorders, urinary diseases, hematological diseases, other dermatoses, and cardiovascular diseases.

Additionally, patients with mucosal involvement of BP (OR 2.443, 95% CI 1.356, 4.440; $p = 0.003$) and hospitalization (OR 4.025, 95% CI 2.289, 7.079; $p < 0.001$) were more likely to have infectious complications. The maximal control doses of oral corticosteroids were higher in the infected group (OR 2.539, 95% CI 1.456, 4.430; $p = 0.001$). Infectious diseases were not related to applying the immunosuppressants or not ($p = 0.062$). The duration of hospitalization in inpatients was also longer in the infected group (27.8 d vs. 18.1 d, $p = 0.010$). The gender distribution ($p = 0.422$), average age ($p = 0.385$) of patients in two groups were similar.

On multivariate analysis, the maximal control dose of corticosteroids (OR 2.539, 95% CI 1.456–4.430, $p = 0.001$), low serum albumin level (OR 2.557, 95% CI 1.283, 5.092, $p = 0.007$), hospitalization (OR 4.025, 95% CI 2.289, 7.079, $p < 0.001$), comorbidities including respiratory disease (OR 4.060, 95% CI, 1.861, 8.858, $p < 0.001$), eye disease (OR 4.431, 95% CI 1.864, 10.532, $p < 0.001$), and diabetes (OR 2.667, 95% CI 1.437, 4.949, $p = 0.002$) remained significant. Osteoarthropathy

TABLE 2 | Sites of infectious complications and pathogens.

Body sites	Total ($N = 81$)	Specimen	Species (numbers of cases)
Skin and perineum	**40**	**Pus**	*Staphylococcus aureus* (4), *Pseudomonas aeruginosa* (3), *Corynebacterium* (2), *Staphylococcus haemolyticus* (2), *Staphylococcus epidermidis* (2), *Enterobacter cloacae* (2), *Enterococcus faecium* (1), *Enterococcus raffinosus* (1), *Staphylococcus intermedius* (1), *Streptococcus pyogenes* (1), β-hemolytic streptococcus (1), *Acinetobacter* (1), *Serratia marcescens* (1), *Proteus mirabilis* (1), *Prevotella melaninogenica* (1), *Actinomyces odontolyticus* (1), *Candida albicans* (1), *Candida parapsilosis* (1), *Candida glabrata* (1), CMV (1)
Mouth	**4**	**Mouth swab**	*Candida albicans* (2)
		Throat swab	*Staphylococcus aureus* (1), *Candida albicans* (1), *Mycoplasma chlamydia* (1)
Respiratory system	**32**	**Sputum**	*Candida albicans* (2), *Acinetobacter baumannii* (1), *Corynebacterium* (1), *Staphylococcus aureus* (1), *Streptococcus pneumonia* (1), *Moraxella catarrhalis* (1)
Pneumonia	23		
Upper respiratory infection	5		
Pulmonary tuberculosis	4		
Urinary system	**8**	**Urine**	*Escherichia coli* (2), *Candida albicans* (2), *Candida tropicalis* (2), *Klebsiella pneumonia* (1), *Enterobacter gergoviae* (1), *Enterococcus faecalis* (1), *Proteus mirabilis* (1)
Digestive system	**4**	**Feces**	*Candida tropicalis* (1)
Hepatitis B	2		
Dysentery	1		
Diarrhea	1		
Blood	**10**	**Blood**	CMV (3), *Staphylococcus aureus* (2), *Enterococcus faecalis* (2), *Enterobacter cloacae* (1), *Staphylococcus haemolyticus* (1), *Listeria monocytogenes* (1), *Serratia marcescens* (1), *Pseudomonas aeruginosa* (1), EBV (1)
Bacteremia	9		
Septic shock	1		
Central nervous system	**1**		

81 of 252 patients with BP had infectious complications. The left column of the table shows the types of infection that occurred in different organ systems, including skin or perineum, mouse, respiratory system, urinary system, digestive system, blood, and central nervous system. The right column revealed the tested specimens and identified pathogens from the body sites of these patients. A patient may have infections at different body sites.

often occurred after the corticosteroid treatment, so it was not regarded as an independent risk factor for developing infectious complications.

The Difference of Infections in Inpatients and Outpatients

Eighty-seven inpatients and 165 outpatients were followed up. Among 81 patients with infectious diseases, 47 (54.0% of the inpatients) of them were inpatients, and 34 (20.6% of the outpatients) outpatients. There was a significantly higher rate of infections in inpatients compared to that of outpatients ($p < 0.001$).

We further analyzed the basic characteristics, all comorbidities, and results of blood tests of the two groups listed previously in **Table 1**, **Figure 1**, and **Supplementary Figure 1**. The comparison was made between the infected group and the control group in inpatients and outpatients, respectively. The characteristics of infected group of inpatients and outpatients were also compared (**Table 3**).

In the inpatient group, old age, high anti-BP180 titer, hyperlipidemia, and respiratory disease were associated with a higher incidence of infectious complications. In the outpatient group, low serum hemoglobin levels, mucosal involvement of BP, comorbidities of diabetes, gastritis, osteoarthropathy, and neurologic disorder were associated with a higher incidence of infectious complications. Compared to the outpatients with infections, the inpatients with infections were treated with a significantly higher control dose of corticosteroids ($p < 0.001$) and had a higher incidence of hyperlipidemia ($p = 0.002$) and respiratory disorders ($p = 0.035$). There is no significant difference in gender and onset-time variation of BP or infection.

The Difference Between Infections in Different Organ Systems

Infections in the respiratory system, hematological system, skin, and mucosa were analyzed, but not in the digestive system and urinary system because of the small sample size (<10). Patients with infection at each site were compared with patients in the control group (uninfected BP patients at the same site).

Respiratory infections were related to advanced age ($p = 0.008$) (**Table 4**). The dose of corticosteroids was associated with an increased tendency of infection, and subgroups analysis showed that the control dose of corticosteroids was significantly correlated with mucocutaneous infections ($p = 0.004$) but not with respiratory ($p = 0.268$) or bacteremia (0.062) (**Table 4**). Mucosal involvement of BP was associated with only hematologic infections ($p < 0.001$) but not the other two infections ($p = 0.068$). The incidence of hospitalization was significantly higher in patients with mucocutaneous infections ($p < 0.001$) or hematologic infections ($p < 0.001$), but not in patients with respiratory infections ($p = 0.067$).

Analysis of all comorbidities and results of blood tests in subgroups confirmed that most of the associated factors were

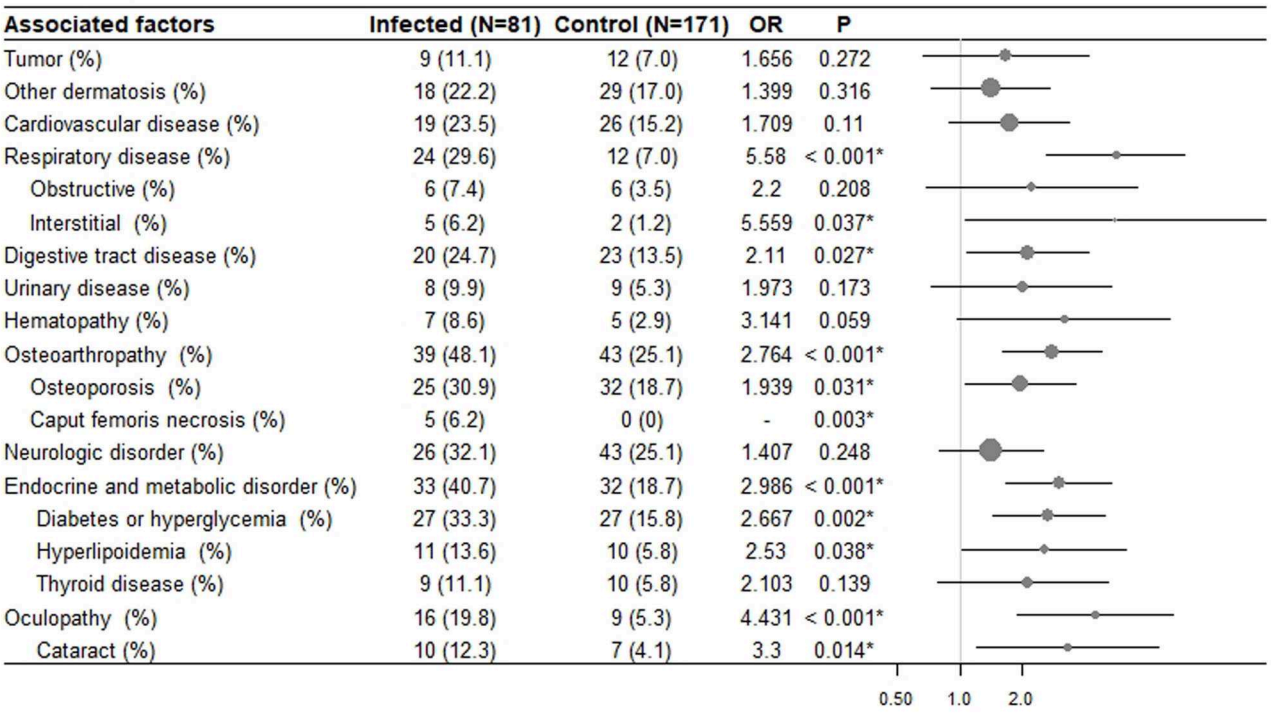

FIGURE 1 | The risk of infection in BP patients with different comorbidities. In all 252 BP patients, 171 had no infectious complication, and 81 had infections. The odds ratios (OR) and *p* values were calculated. *Denotes statistical significance ($p < 0.05$). Forest plots show odds ratios of different comorbidities with a 95% confidence interval.

the same as previously analyzed. We listed three comorbidities, serum hemoglobin level, and BP180 antibody titer in **Table 4**, to reveal the relationship between the infection and the abnormality at the same site. We found mucocutaneous infections were associated with other dermatoses ($p = 0.043$); respiratory infections were related to respiratory disease ($p = 0.008$), and hematologic infections were associated with low serum hemoglobin level ($p = 0.034$).

DISCUSSION

In our study, we have shown that 32.1% of patients with bullous pemphigoid were affected by infectious complications. The median duration before infection was 5 months, with 61.7% occurring in the first year after BP diagnosis. Mucocutaneous (17.5%) and respiratory infections (12.7%) were the most frequent, followed by bacteremia (4.0%). The most common pathogens were *S. aureus* and *C. albicans*. Factors associated with developing infections on univariate analysis were the comorbidities of multiple systems, mucosal involvement of BP, more extended periods of hospitalization, higher maximal control doses of corticosteroid, and a lower level of serum albumin. On multivariate analysis, a higher dose of corticosteroid, a lower level of serum albumin, the experience of hospitalization, and comorbidities, including respiratory disease, eye disease, and diabetes, remained significant.

The inpatients were more vulnerable to infectious complications than outpatients (54 vs. 20.6%, $p < 0.001$). A plausible explanation could be that the inpatients had a higher maximal control dose of corticosteroid and a higher frequency of respiratory diseases—both are significant risk factors of infections in BP. On subgroup analysis, mucocutaneous infections were associated with corticosteroids and other dermatoses. As predicted, respiratory infections were related to respiratory disease. Hematologic infections were associated with low serum hemoglobin levels, although the significance and mechanism remain to be investigated in the future.

Interestingly, mucosal involvement of BP was significantly correlated with hematologic infections or sepsis, which could contribute to higher mortality of BP patients. Moreover, we found that the ratio of patients with mucosal involvement of BP was significantly higher in the infected outpatients than in the uninfected outpatients with BP. Kridin and Bergman have shown that a majority of mucosal involvements of BP occur in the mouth and are related to extensive cutaneous disease, lower peripheral eosinophilia, and more aggressive treatment (20). In our study, the association between infectious complications and increased doses of corticosteroids was also significant, suggesting that the correlation between mucosal involvement of BP and infection is dependent on treatment.

As the BP patients with cardiovascular diseases, dementia, and stroke proved to be at a higher risk of mortality (21, 22), we further analyzed the correlation between comorbidities and

TABLE 3 | Infectious complications in BP inpatients and outpatients.

Group of patients	Inpatients ($N = 87$)			Outpatients ($N = 165$)			P
	Infected	Control	p	Infected	Control	p	
Number (%)	47 (54.0)	40 (46.0)	–	34 (20.6)	131 (79.4)	–	<0.001*
Gender (M/F)	31/16	21/19	0.273	16/18	69/60	0.565	0.112
Age of BP onset/y	66.9 ± 1.9 67.1	59.3 ± 2.6, 60.2	0.020*	70.6 ± 3.2, 76.9	68.9 ± 1.3, 70.9	0.249	0.098
Duration before diagnosis/m	10.7 ± 3.0, 4.0	6.1 ± 1.3, 3.0	0.928	7.8 ± 4.0, 3.0	9.9 ± 1.8, 3.0	0.430	0.469
Duration before infection/m	14.4 ± 3.2, 5.2	–	–	15.5 ± 3.3, 5.2	–	–	0.105
Duration of Hospitalization/d	27.8 ± 3.1, 26.0	18.1 ± 1.7, 17.0	0.010*	–	–	–	–
Mucosal involvement of BP	21 (44.7%)	14 (35.0%)	0.381	11 (32.4%)	20 (15.3%)	0.044*	0.271
Control dose of corticosteroid (mg/d)	53.3 ± 6.4, 40.0	42.4 ± 4.3, 40.0	0.534	26.8 ± 3.1, 30.0	24.3 ± 2.4, 20.0	0.213	<0.001*
Auxiliary exam							
Anti-BP180	75.1 ± 8.6, 74.0	47.7 ± 8.7, 33.0	0.015*	64.9 ± 12.0, 23.0	57.7 ± 5.2, 43.0	0.699	0.300
Serum Hb	131.8 ± 2.9, 138.0	130.5 ± 3.2, 132.0	0.563	128.9 ± 3.3, 134.0	136.8 ± 1.8, 135.0	0.033*	0.372
Serum Alb	34.9 ± 1.1, 36.0	37.4 ± 0.8, 37.0	0.071	41.0 ± 1.8, 39.0	40.9 ± 0.7, 41.0	0.215	0.014*
Comorbidities							
Diabetes	17 (36.2%)	10 (25.0%)	0.262	10 (29.4%)	17 (13.0%)	0.021*	0.524
Hyperlipidemia	11 (23.4%)	2 (5.0%)	0.018*	0 (0.0%)	8 (6.1%)	0.208	0.002*
Gastritis	1 (2.1%)	2 (5.0%)	0.592	4 (11.8%)	2 (1.5%)	0.017*	0.156
Osteoarthropathy	22 (46.8%)	14 (35.0%)	0.265	17 (50.0%)	29 (22.1%)	0.001*	0.777
Respiratory disease	15 (31.9%)	2 (5.0%)	0.002*	4 (11.8%)	10 (7.6%)	0.490	0.035*
Neurologic disorder	12 (25.5%)	14 (35.0%)	0.336	14 (11.8%)	29 (22.1%)	0.024*	0.137

*For inpatients and outpatients, the difference between patients with infections (infected) and without infection (control) was compared, respectively. The p values were calculated in columns 4 and 8, respectively. The difference between the infected group of inpatients and outpatients was also estimated with p values (last column) (*denotes statistical significance or $p < 0.05$). BP, bullous pemphigoid.*

infectious complications. Our results indicate that infections are significantly higher in BP patients with comorbidities such as diabetes and respiratory diseases. A nationwide study from the USA found that a higher number of chronic conditions (RA, SLE, or type I diabetes) was a risk factor of severe infections in BP patients (18). A retrospective study from Singapore found that functional impairment (Karnofsky score <60, CCIS ≥6) and dementia are risk factors of infection in a cohort of 97 BP patients (17). The severity of disease and diabetes have also been reported to be directly related to more infections in bullous diseases (23, 24). Nevertheless, we cannot exclude the possibility that patients with infectious complications tend to receive more medical examinations with more detailed documentation of medical history, which may contribute to the higher number of comorbidities in BP patients.

Notably, we found that lower serum Alb level and higher BP180 antibody titer were significantly associated with the development of infectious complications in BP patients. A higher BP antibody level could cause a more severe disruption of the skin barrier function, which could lead to more infections. Our results indicated that a higher titer BP180 antibody was associated with respiratory and hematologic infections. The serum alb level is a marker of the nutrition level. Research published recently pointed out that patients at risk of malnutrition were more likely to have a healthcare-associated infection, with malnutrition assessed by BMI, recent weight loss, and dietary intake (25). A case report and retrospective analysis from Japan discovered that higher BP180 antibody titer resistant to corticosteroids treatment was a risk factor for developing CMV infection in BP patients (26). The infection by Varicella Zoster virus (VSV) that also belongs to the family of herpes virus as CMV was reported to enhance BP180 antibody production (27). Additionally, reduction in serum WBC, PLT, and increase in ALT have also been reported to be risk factors (26).

Some therapies, particularly corticosteroids, could lead to more infectious complications in BP patients. A retrospective study in Mayo Clinic revealed that all patients with autoimmune bullous diseases and taking systemic corticosteroids had an infection during the follow-up (12). The risk of using topical corticosteroids should not be neglected, especially in patients with diabetes and mucocutaneous infection (13). Case reports and literature reviews have demonstrated that corticosteroids and ibrutinib may be associated with opportunistic fungal and virus infections (28–31). The aforementioned nationwide study in the USA also pointed out that severe infections in inpatients with pemphigus and pemphigoid were associated with prolonged hospitalization and increased mortality (18). In our preliminary clinical observations, the choice of hospitalization and the duration in the hospital were not risk factors for infection in bullous pemphigoid, potentially because infectious complications can prolong hospital stay in the first place.

Other risk factors for infections in BP patients include female, non-white race, and poor economic condition, as described in the previous studies (18). The circulating anti-centrosome antibody has also been reported associated with infection (32).

TABLE 4 | Infectious complications in different organ systems, including mucocutaneous, respiratory, and blood.

	Mucocutaneous			Respiratory			Blood		
	Infected (*N* = 44)	Control	*p* (*N* = 208)	Infected (*N* = 32)	Control	*p* (*N* = 220)	Infected (*N* = 10)	Control (*N* = 242)	*p*
Gender (M/F)	1.59	1.05	0.351	1.46	1.19	0.596	0.30	1.28	0.118
Age of BP onset/year	64.6 ± 2.2, 65.1	67.9 ± 1.1, 70.5	0.140	72.1 ± 3.5, 76.9	66.2 ± 1.1, 67.2	0.008*	63.6 ± 2.5, 64.4	67.5 ± 1.0, 70.4	0.202
Duration before diagnosis/months	9.4 ± 3.7, 3.0	9.1 ± 1.3, 3.0	0.224	10.4 ± 3.6, 5.0	8.9 ± 1.3, 3.0	0.707	3.3 ± 0.9, 3.0	9.4 ± 1.3, 3.0	0.398
Duration before infection/months	16.3 ± 3.4, 3.7	-	-	15.0 ± 3.2, 5.1	-	-	2.1 ± 0.3, 2.1	-	-
Control dose of corticosteroids (mg/day)	49.2 ± 7.1, 40.0	29.8 ± 1.9, 30.0	0.004*	34.8 ± 3.9, 33.8	33.1 ± 2.3, 30.0	0.268	69.8 ± 25.0, 40.0	31.8 ± 1.9, 30.0	0.062
Mucosal involvement of BP (%)	17 (38.6)	49 (23.6)	0.068	13 (40.6)	53 (24.1)	0.068	9 (90.0)	57 (23.6)	<0.001*
Auxiliary exam									
Hb < 130 g/L (%)	27 (61.4)	106 (51.0)	0.960	17 (53.1)	116 (52.7)	0.269	3 (30.0)	130 (53.7)	0.034*
Anti-BP180 > 50 U/L (%)	18 (40.9)	62 (29.8)	0.747	16 (50.0)	64 (29.1)	0.023*	8 (80.0)	72 (29.8)	0.002*
Comorbidities									
Other dermatosis (%)	13 (29.5)	34 (16.3)	0.043*	7 (21.9)	40 (18.2)	0.625	2 (20.0)	45 (18.6)	1.000
Respiratory diseases (%)	10 (22.7)	21 (10.1)	0.021*	9 (28.1)	22 (10.0)	0.008*	5 (50.0)	26 (10.7)	0.003*
Anemia (%)	5 (11.4)	5 (2.4)	0.017*	4 (12.5)	6 (3.0)	0.026*	2 (20.0)	8 (3.3)	0.054
Hospitalization									
Number (%)	28 (63.6)	54 (26.1)	<0.001*	15 (46.9)	67 (30.5)	0.067	9 (81.2)	73 (30.2)	<0.001*
Duration/day	27.9 ± 3.7, 25.5	20.7 ± 2.0, 17.0	0.045*	26.9 ± 5.7, 21.0	22.4 ± 1.9, 19.0	0.556	26.8 ± 5.6, 28.0	22.7 ± 2.0, 18.0	0.350

*In each type of infection, the difference between patients with infection (infected) and without infection (control) was calculated as p-values, respectively. *Denotes statistical significance or p < 0.05.*

Interestingly, two previous retrospective analyses have shown that the incidence of pneumocystis pneumonia (PCP) infection was lower than expected in patients with the autoimmune bullous disease (33, 34).

CONCLUSION AND LIMITATIONS

BP patients have a high risk of infectious complications, which are associated with mucosal involvement of BP, more comorbidities, a higher dose of corticosteroid, and a lower level of serum albumin. The inpatients have a higher risk of infection than the outpatients. The main limitation of this study originated from the retrospective nature of data collection. The factors that may affect the interpretation of results are the detailedness of documentation of hematological findings, the accuracy of recording of infected sites, and comprehensiveness of screenings for pathogens. Another limitation could be that most of our patients were residents from the north of China, although they were from different provinces. However, our study comprised a relatively large cohort of both inpatients and outpatients and described the pathogens of infectious complications. Infectious complications were analyzed further in subgroups as mucocutaneous, respiratory, and hematological infections. To our knowledge, this is the first study for characterizing infectious complications in BP outpatients. Our results should provide insight into better management of BP patients during the long-term, chronic disease course. In the future, a multicentric study with a larger sample size will be needed to verify our study.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Peking Union Medical College Hospital (S-K965). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JC performed the data analysis and drafted the manuscript. XM and WZ revised and edited the manuscript. BZ, XC, CY, and ZZ collected information and were responsible for data curation. HJ provided resources and information. LL conceived the idea, revised, and edited the manuscript. All authors read and made final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01607/full#supplementary-material

Supplementary Figure 1 | Results of serum laboratory tests. The values of the variables, as indicated on the bottom of each figure, were plotted. The difference of uninfected patients (control) and infected patients were compared, as indicated with the P-value on the top of each figure. WBC, white blood cell; Ly#, cell count of lymphocyte; Ly%, percentage of lymphocyte; Eos#, cell count of eosinophilia; Eos%, percentage of eosinophilia; Alb, serum albumin; PAB, serum pre-albumin; Hb, hemoglobin.

Supplementary Table 1 | Application of immunosuppressant in all BP patients. The numbers (*n*) and percentages(%) of patients treated with each of the immunosuppressants were calculated(left column). The Right column shows the numbers(*n*) and percentages(%) of patients treated with one, two, or three types of immunosuppressants, respectively. MTX, methotrexate; MMF, Mycophenolate mofetil; CTX, cyclophosphamide; CsA, cyclosporine.

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MINI REVIEW
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Recent Advances in the Use of Exosomes in Sjögren's Syndrome

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Sjögren's syndrome (SS) is a chronic autoimmune disorder of the exocrine glands mediated by lymphocytic infiltrates damaging the body tissues and affecting the life quality of patients. Although traditional methods of diagnosis and treatment for SS are effective, in the time of personalized medicine, new biomarkers, and novel approaches are required for the detection and treatment of SS. Exosomes represent an emerging field in the discovery of biomarkers and the management of SS. Exosomes, a subtype of extracellular vesicles, are secreted by various cell types and can be found in most bodily fluids. Exosomes are packed with cytokines and other proteins, bioactive lipids, and nucleic acids (mRNA, circular RNA, non-coding RNA, tRNA, microRNA, genomic DNA, and ssDNA), and transport such cargo between cells. Evidence has indicated that exosomes may play roles in processes such as the modulation of the immune response and activation of inflammation. Moreover, due to features such as stability, low immunogenicity and toxicity, long half-life, and the capacity to penetrate the blood-brain barrier, exosomes have also emerged as therapeutic tools for SS. In this review, we summarize existing literature regarding the biogenesis, isolation, and function of exosomes, specifically focusing on exosomes as novel biomarkers and their potential therapeutic uses in SS.

Keywords: Sjögren's syndrome, exosomes, immune response, biomarkers, treatment

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INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune disorder of the exocrine glands. It is characterized by lymphocytic infiltration in the salivary and lacrimal glands (LGs) resulting in oral and eye dryness. Extraglandular manifestations such as musculoskeletal pain, fatigue, and systemic features also develop in a significant percentage of patients. This exocrinopathy can occur alone (primary Sjögren's syndrome, pSS) or secondary to another autoimmune disease such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and systemic sclerosis (SSc). The antinuclear antibody (ANA) is the most frequently detected autoantibody in SS, while anti-Ro/SSA and anti-La/SSB are the most specific prognostic markers (1–3). The prevalence of SS is 0.29–0.77% overall and 3–4% among the elderly. The ratio of male to female cases is 1:9, and the average age of onset is over 50 years. 5% to 10% of the patients can develop non-Hodgkin's lymphoma, the most serious complication of SS, within 10 to 15 years of follow-up (4, 5). Despite extensive research on the underlying cause of SS, the pathogenesis remains obscure. Multiple factors, including the environment and the immune system, may contribute to the development of this disease.

In the last decade, researchers have focused their attention on the release of extracellular vesicles (EVs). These double lipid bilayer-enclosed membranous vesicles are produced and discharged from almost all cells and represent more than the casual dispersal of cellular "dust" (6). Further, EVs deliver complex chemical messages over long distances (7). Exosomes, the most well-known and studied subtype of EVs, were first described as nanoscale vesicles derived from various normal and neoplastic cell lines in the 1980s (8, 9). These endosome-derived nanovesicles have a characteristic cup-shaped morphology as observed under electron microscopy, with a diameter of 30–100 nm and a density between 1.13 g/ml and 1.19 g/ml. They exist in numerous bodily fluids, including serum, saliva, urine, cerebrospinal fluid, milk, and tears, under both normal and pathological conditions (10, 11). Exosomes are packed with various components and have the capacity of inducing functional responses in recipient cells (12–15). Through the transfer of bioactive molecules between cells, exosomes mediate intercellular signaling and participate in various physiological and pathological processes (16). The involvement of exosomes in the development and treatment of autoimmune diseases has also been extensively researched (17, 18). Lee et al. demonstrated that circulating exosomes in patients with SLE could be associated with disease activity and might therefore serve as biomarkers of disease activity (19). Kimura et al. found that circulating exosomes suppressed the induction of regulatory T cells *via* let-7i-mediated blockade of the IGF1R/TGFBR1 pathway in multiple sclerosis (20). Few reviews have investigated and summarized the functions of exosomes in SS. In this review, we will focus on the recent advances regarding exosomes in SS and their potential as biomarkers and therapeutic tools.

EXOSOMES

Exosome Biogenesis and Isolation Methods

The generation of exosomes is initiated by invagination of the plasma membrane to form endocytic vesicles. When these newly formed endosomes mature, depressions in the endosomal membrane take place, and intraluminal vesicles are produced. Intraluminal vesicles are further transformed into multivesicular bodies (MVBs) with a dynamic subcellular structure, also known as late endosomes. MVBs then merge with the plasma membrane and release the vesicles contained within, called exosomes. Exosome biogenesis is complex and tightly regulated by multiple factors. The endosomal sorting complex required for transport (ESCRT) is the principal protein family governing the synthesis of exosomes. Downregulation of ESCRT-0 and ESCRT-0 proteins decrease exosome secretion. Conversely, depletion of ESCRT-I proteins increase exosome production. Moreover, exosomes can be generated without ESCRT proteins, and ESCRT-independent machinery may contribute to the sorting of cargo into exosomes. Lipids also play a crucial role in the biogenesis and transport of exosomes. Several other proteins, including GTPase proteins and lactadherin, are also involved in the biogenesis of exosomes (16, 21, 22). Nevertheless, mechanisms of exosome biogenesis and secretion require further elucidation.

Exosomes are secreted into biological fluids which contain other vesicle types such as microvesicles and apoptotic bodies. It is therefore necessary to isolate exosomes from contaminating material. The isolation of pure exosomes is essential for understanding their mechanisms of action and potential applications. Several methods have been adopted for the isolation for exosomes: differential centrifugation, ExoQuick^{TM} extraction kits, sucrose density gradient ultracentrifugation, and immunoaffinity sedimentation (21). These methods may have certain limitations, such as low yield and purity. Microfluidics-based technologies have recently become available for the isolation, detection, and analysis of exosomes and do not have the above-mentioned limitations (16).

Accurate evaluation of the physicochemical characteristics of exosomes, such as size, shape, and density, is crucial for exploring the biological interactions of these vesicles. Western blotting, enzyme-linked immunosorbent assay (ELISA), real-time quantitative polymerase chain reaction (RT-qPCR), dynamic light scattering (DLS), fluorescence-based detection, nanoparticle tracking analysis (NTA), atomic force microscopy (AFM), and transmission electron microscopy (TEM) are commonly used techniques for exosome characterization (23, 24). Western blotting and ELISA are used for the identification of intra-vesicular or membrane protein markers (25), while RT-qPCR is used for the detection of exosome-related RNA (26). NTA, AFM, and TEM have been developed to determine the size, density, morphology, and composition of exosomes (27, 28). Recently, a new technique, tunable resistive pulse sensing (TRPS), has been used to measure the size distribution and concentration of exosomes (29). To discriminate between exosomes from normal and pathological cells, considering their inherent heterogeneity, we need to combine quantification techniques. This will open up new avenues for exosome detection and characterization.

Composition and Function of Exosomes

Exosomes have a lipid bilayer structure and are released upon fusion of the MVB with the plasma membrane (30–32). Exosomes contain various proteins (e.g., cytokines, GTPases, Alix, TSG101, tetraspanins, heat shock proteins, and integrins), lipids (e.g., phosphoglycerides, cholesterol), and nucleic acids (e.g., mRNA, circular RNA, non-coding RNA, tRNA, microRNA, genomic DNA, and ssDNA) (33–38). Due to their lipid bilayer, genetic information and other transported components are protected from degradation (31). Exosomes are secreted by various immune cells [e.g., T cells, B cells, dendritic cells (DCs), and macrophages] and non-immune cells (39). Once released, exosomes can interact with specific recipient cells based on the expression of adhesion molecules, such as phosphatidylserine receptors, integrins, and glycans on the exosome surface (40, 41). Thus, information can be transmitted to target cells *via* exosomes.

The existence of EVs had been reported as early as 1946 (42), and De Broe described the release of these "membrane fragments" as a general characteristic of viable cells in 1977 (43). In 1983, a major discovery by Harding and Johnstone revealed

that transferrin receptors were associated small 50 nm-sized vesicles. Through endocytosis and recycling, these vesicles were released from maturing blood reticulocytes into the extracellular space. The term "exosome" was coined by Rose Johnstone to describe these EVs (16). In 1996, researchers found that Epstein-Barr (EB) virus-transformed B lymphocytes had the capacity of releasing exosomes, inducing major histocompatibility complex (MHC) class II-restricted T-cell responses (44). In the early days of EV research, exosomes were simply considered as cellular waste disposal units. In more recent years, however, exosomes have been intensively researched and have been shown to act as mediators of immune stimulation and modulation (45). Exosomes regulate multiple immune processes, including antigen presentation, T-cell activation and polarization, and immune suppression (46, 47). Immune cell-derived exosomes have been studied extensively. For example, MHC-I and MHC-II molecule-carrying exosomes derived from antigen-presenting cells stimulate CD8 + and CD4 + T cells, respectively (48). Further, exosomes secreted from macrophages infected with bacteria had pro-inflammatory effects on naïve macrophages, promoting the maturation of DCs (49). It should be noted that the release of exosomes by DCs and B lymphocytes is increased after cognate T cell interactions, indicating that the secretion of exosomes by immune cells could be regulated by the cellular environment (50, 51). Exosomes secreted by non-immune cells, such as mesenchymal stem cells (MSC) and tumor cells, have also gained attention. MSC-derived exosomes are capable of enhancing the differentiation of immunosuppressive cells and inhibiting the proliferation of natural killer (NK) cells and T cells (52). Recent research has reported that exosomes from bone marrow-derived mesenchymal stem cells (BMSC) regulate the polarization of macrophages in rat models (53). In addition, exosomes derived from tumor cells can inhibit the activation of T cells *via* programmed death-ligand 1 (PD-L1) (54). Among exosome-associated bioactive components, microRNAs (miRNAs) not only modulate gene expression in immune cells, but also have immunological functions (55, 56). Okoye et al. suggest that miRNA-containing exosomes secreted from primary regulatory T cells suppress Th1 cell responses (57). Ismail et al. found that macrophage-derived exosomal miR-223 induced the differentiation of recipient monocytes (58). Another study showed that miR-223 promoted the invasion of breast cancer cells *via* the Mef2c-β-catenin pathway (59). Other functions of exosomes have also been investigated, including regulation of the incorporation of neurons and glial cells in the central nervous system (60, 61) and thrombosis in the cardiovascular system (62–64). A previous review summarized the involvement of exosomes in: (1) protection against viruses and bacteria; (2) regulation of tumor immunity; (3) mediation of immune suppression by tumor cells (65). In general, the function of exosomes depends on the status of host cells and tissue. Studies have shown that exosomes play significant roles in angiogenesis, antigen presentation, apoptosis, coagulation, inflammation, and intercellular communication through the transfer of bioactive molecules such as RNA and proteins. Further, exosomes are involved in both normal and pathological processes, including cancer, infections, and autoimmune diseases.

Exosome carrying specific molecules of interest could act as potential biomarkers. Exosomal biomarkers can be divided into three groups: tumor-derived exosomes, exosomal surface proteins, and exosomal nucleic acids (66), and these indicators can provide insightful information for the early diagnosis of cancer and other diseases. For example, exosomes containing proteoglycan glypican-1 (GP1) may be potential biomarkers for pancreatic cancer (67). Exosomes loaded with CD81 have a potential role in the diagnosis of hepatitis C and the evaluation of treatment responses (68). Exosomes carrying a specific kind of phosphorylated amyloid peptides are promising biomarkers for Alzheimer's disease (69). Some unique characteristics of exosomes have attracted the interest of researchers, including their stability under long-term storage, low immunogenicity and toxicity, their ability to protect encapsulated components, and their capacity for penetration of the blood-brain barrier (BBB) (70–73). Thus, exosomes could potentially be used as nanocarriers for various nucleic acids, proteins, and small molecular drugs (74). Some antineoplastic agents, such as doxorubicin and paclitaxel, could be encapsulated and delivered via exosomes to treat brain tumors (75, 76). Tian et al. revealed that curcumin-carrying engineered exosomes induced the suppression of the inflammatory response and cellular apoptosis in lesion regions of ischemic brains (77).

There are various studues investigating exosomes in autoimmune diseases, among which studies of rheumatoid arthritis (RA) have been the most thorough. With regard to pathogenesis, in the synovium of RA patients, synoviocyte-derived exosomes, which contain citrullinated autoantigens, may promote synovitis and cartilage damage (78, 79). In contrast, exosomes from neutrophils that have infiltrated into inflamed joints are protective factors for chondrocytes (80). From the perspective of treatment, BMSC-secreted exosomal miR-192-5p can delay inflammation in RA (81). Mesenchymal cell–derived miRNA-150-5p–containing exosomes and MSCs-derived miRNA-124a-overexpressing exosomes are also expected to be involved in potential therapeutic strategies for RA patients (82, 83). Information about the role of exosomes in the pathogenesis or their possible use for treatment of other autoimmune diseases has been scarce in comparison to RA. It has been suggested that exosomes from inflamed intestinal cells and renal tissue have pathogenic roles in ulcerative colitis and lupus nephritis, respectively (84, 85). Lu et al. showed that BMSC-derived exosomes carrying miR-223-3p attenuated autoimmune hepatitis in a mouse model (86). Neutrophil-produced exosomes from systemic sclerosis patients have the ability to inhibit the proliferation and migration of endothelial cells (87).

EXOSOMES IN SJÖGREN'S SYNDROME

Role of Exosomes in the Pathogenesis of SS and as Potential Biomarkers

In 2005, Kapsogeorgou et al. reported that salivary gland epithelial cell (SGEC) lines from SS patients secreted significant amounts of exosomal vesicles, similar to those from non-SS subjects. These SGEC-derived exosomes contained detectable

TABLE 1 | A selective overview of studies reporting exosomes in Sjögren's syndrome.

Articles	Origin of exosomes	Exosome components	Role in pathology	As a biomarker	Potent therapeutic effect
(88)	SGECs		+		
(89)	B cells	ebv-miR-BART13-3p	+		
(90)	T cells	miR-142-3p	+		
(93)	SGECs, lacrimal gland cells			+	
(94)	SGECs	miRNAs		+	
(95)	SGECs	miRNAs		+	
(96)	DCs				+
(97)	DCs				+
(98)	MSCs				+
(100)	Placenta tissue	miRNAs of C19MC			+
(87)	human umbilical cord MSCs				+

SGECs, salivary gland epithelial cells; DCs, dendritic cells; MSCs, mesenchymal stem cells; miRNA, microRNA; C19MC, chromosome 19 microRNA cluste.

amounts of epithelial-specific cytoskeletal proteins, as well as anti-Ro/SSA, anti-La/SSB, and Sm ribonucleoproteins. Although secretion was not restricted to SS-derived cells, this was the first time that SS-specific autoantigens were detected in exosomes, indicating that exosomes may participate in the presentation of intracellular autoantigens to autoreactive lymphocytes, as part of the development of SS (88). Another study showed that a functional EB virus miRNA, ebv-miR-BART13, can be transferred from B cells to SGECs, affecting salivary secretion (89). More recently, Cortes-Troncoso et al. suggested that T cell-derived exosomes containing miR-142-3p may be a pathogenic trigger of SS. When transferred into SGECs, miR-142-3p-carrying exosomes can affect intracellular Ca^{2+} signaling and decrease cyclic adenosine monophosphate (cAMP) production, thereby leading to glandular cell dysfunction (90). At present, studies of exosomes in SS mainly concentrate on tears and saliva (**Table 1**), as such fluid samples can easily be obtained using a simple, non-invasive, and safe method. Because SS is a disease affecting multiple organ systems, investigation of exosomes in other tissues and organs is still required.

The international consensus criteria for SS includes ocular symptoms, oral symptoms, objective evidence of dry eyes and salivary gland involvement, as well as laboratory test abnormalities (91). The presence of ANA has some merit for the detection of SS, but 31.7% of healthy individuals may also be positive for ANA (92). Rheumatoid factor (RF) is not specific to SS, as it is also upregulated in other autoimmune diseases, especially RA. Anti-Ro/SSA antibodies have good specificity and can be found in two-thirds of SS patients, often at the same time as anti-La/SSB antibodies (91). However, sometimes during the early stage of the disease, patient symptoms are not typical, and even the serological examination is not positive. Therefore, a more accurate diagnostic method is required. Aqrawi et al. isolated EVs (including exosomes and microvesicles) from saliva and tear fluids of patients with SS and utilized liquid chromatography-mass spectrometry (LC-MS) for the detection of potential biomarkers (93). Michael et al. were the first to isolate exosomal miRNAs from the parotid saliva of SS patients, proposing that the miRNA content of salivary exosomes could provide markers for the diagnosis of various salivary gland diseases, such as SS (94). Similarly, Alevizos et al. showed that salivary gland miRNA expression patterns precisely distinguished SS patients from control subjects, suggestive of the potential of miRNA for the detection of inflammation or salivary gland dysfunction in SS (95). Despite these promising findings, there is not enough evidence for the use of exosomes or exosomal miRNAs as reliable markers for SS. Future experiments may refute some of the current findings. Moreover, the use of exosomes for evaluating SS disease activity and prognosis has not yet been investigated in studies (**Figure 1**). Thus, further research is required to confirm the potential roles of exosomes or exosomal miRNAs as robust, specific, and sensitive biomarkers for SS.

Exosomes as Therapeutic Tools for SS

The management of SS is long-term and complex. Saliva substitutes and artificial tears could be used to relieve symptoms. Non-steroidal anti-inflammatory drugs (NSAIDs), hydroxychloroquine, and corticosteroids are effective for the treatment of SS. Other powerful immunosuppressants, such as methotrexate, mycophenolate mofetil, and biological agents, are also required (91). However, long-term use of these drugs can cause a number of adverse effects. Fortunately, exosomes have been intensively studied for their potential use in autoimmune diseases. Kim et al. suggested that injection of exosomes secreted from DCs treated with interleukin-10 (IL-10) inhibited the onset of collagen-induced arthritis in a mouse model and reduced the severity of arthritis (96). Exosomes derived from indoleamine-expressing DCs had immunosuppressive and anti-inflammatory effects in an arthritis model (97). Bai et al. reported that exosomes from MSCs efficiently attenuated autoimmune uveitis in a murine model (98).

A study by Li et al. has demonstrated that administered exosomes derived from human umbilical cord MSCs efficiently eased ophthalmitis in a model of human SS (99). Bullerdiek et al. reported that analogs of chromosome 19 miRNA cluster

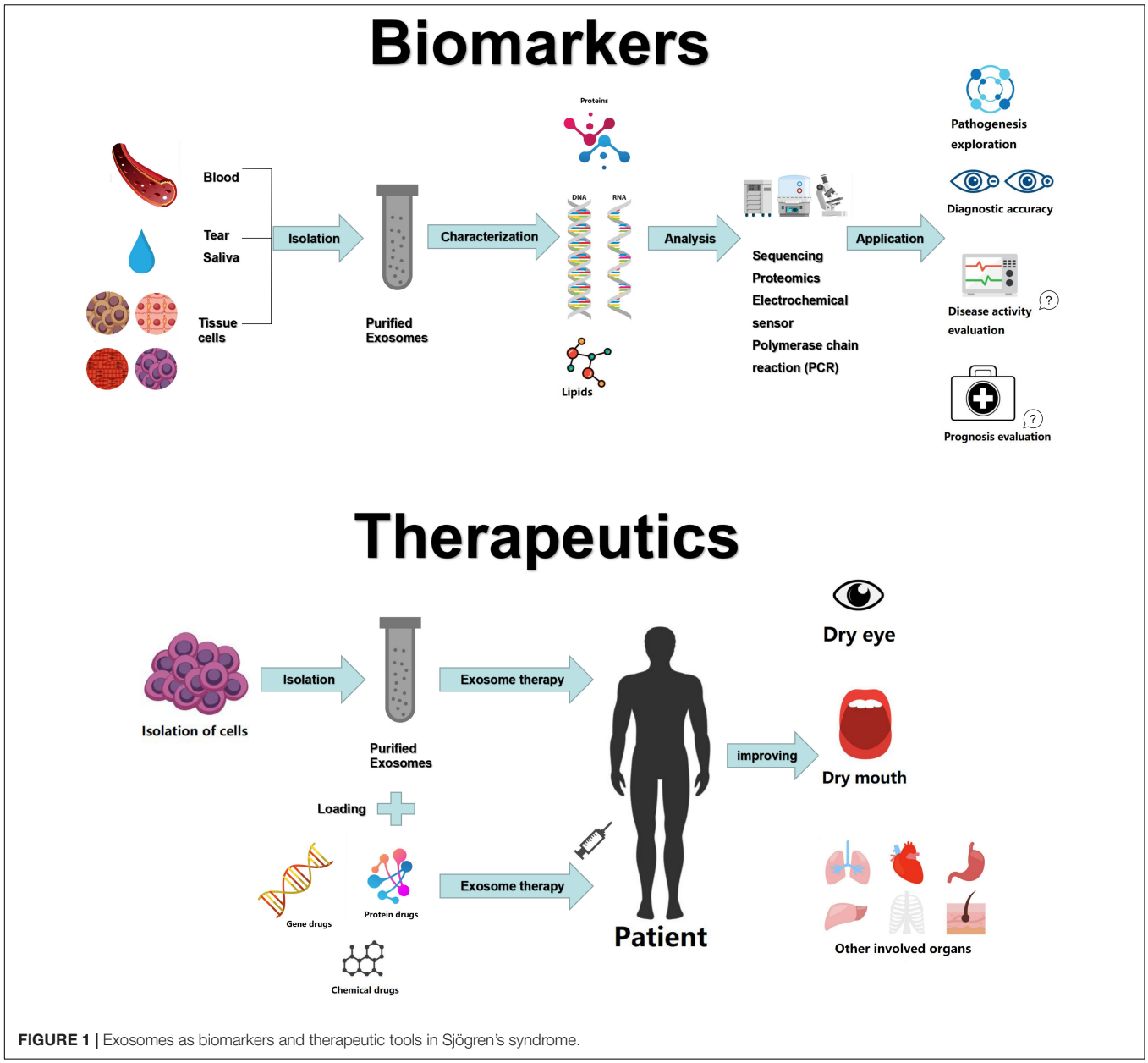

FIGURE 1 | Exosomes as biomarkers and therapeutic tools in Sjögren's syndrome.

(C19MC)-derived miRNAs could be applied in clinical practice for autoimmune conditions such as SS (100). Ocular involvement is one of the main manifestations of SS. The most commonly used treatment for eye disease is topical instillation of eye drops. However, there are some limitations, including quick clearance and low biological activity. Due to their highly desired qualities as drug delivery vehicles, exosomes can be used for the delivery peptides or synthetic drugs for eye disease (101, 102). MSC-exos carrying miRNA-126 could reduce hyperglycemia-induced retinal inflammation by inhibiting the high-mobility group box 1 signaling pathway (103). Exosome-carried adeno-associated virus type 2 showed high efficiency in retinal transduction (104). Therefore, MSC-exos may presumably provide a curative option for SS-associated dry eyes. While, exosomes have shown promising results for potential therapeutic applications, most of these therapeutic effects have only been observed in the experimental stage, and there is a long way to go before reaching large-scale clinical application.

CONCLUSION AND PERSPECTIVES

For decades, researchers have been struggling to develop superior diagnostic and treatment methods for patients with SS. Accumulating evidence has indicated that exosomes may play an important role in the pathophysiology of autoimmune disorders. In this review, we have summarized exosome-mediated effects mediated in SS, the potential of exosomes as biomarkers, as well

as their potential therapeutic uses. Nevertheless, gaps remain in the understanding of exosome biogenesis and action. The fundamental mechanisms of exosomes utilized as biomarkers and therapeutic nanocarriers in SS and other autoimmune diseases are not fully understood. In the future, the use of exosomes for SS and other autoimmune diseases will face several challenges that will require further detailed exploration. First, methods for the detection, separation, and purification of exosomes and exosomal miRNA are relatively cumbersome and complicated at present. Thus, there is a need for simplified, cost-effective, and reproducible techniques. Moreover, appropriate production and storage methods for exosomes are critical for preserving their biological activity and are thus essential for obtaining high-quality exosomes. Existing methods are more or less insufficient in obtaining and preserving high yields of purified exosomes. In addition, it is important to establish robust ways to evaluate the effects of exosomal treatment *in vivo*. Despite challenges in the use of exosomes, these vesicles have shown great potential within the biomedical field. As technology advances, the above-mentioned limitations will be resolved, and exosomes may be utilized for novel and advanced therapies. Altogether, both basic and applied research on exosomes in SS is still at an early stage, requiring further investigation.

AUTHOR CONTRIBUTIONS

YH, RL, GY, and QX designed and revised the review manuscript, and approved the final manuscript. YH and RL wrote the review manuscript. SY and SL helped in finding references. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Immunomodulatory Effects of Mesenchymal Stem Cells and Mesenchymal Stem Cell-Derived Extracellular Vesicles in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects the joints and other organs for which there is currently no effective treatment. Mesenchymal stem cells (MSCs) have therapeutic potential due to their immunomodulatory and differentiation effects. While extensive experimental studies and clinical trials have demonstrated the effects of MSCs in various diseases, MSCs have been found to cause abnormal differentiation and tumor formation. Therefore, extracellular vesicles derived from MSCs (MSC-EVs) are more effective, less toxic, and more stable than the parental cells. MSC-EVs transfer various nucleic acids, proteins, and lipids from parent cells to recipient cells, and thus participate in chronic inflammatory and immune processes. In this review, we summarize the properties and biological functions of MSCs and MSC-EVs in RA. Improvement in our understanding of the mechanisms underlying MSC and MSC-EVs in RA provides an insight into potential biomarkers and therapeutic strategies for RA.

Keywords: rheumatoid arthritis, mesenchymal stem cells, extracellular vesicles, exosomes, microRNAs

INTRODUCTION

Rheumatoid arthritis (RA) is a prevalent systemic autoimmune disease characterized by progressive joint destruction, and 50% of RA patients also have extra-articular involvement, including the heart, lungs, eyes, and blood (1, 2). Globally, the overall incidence of RA is 40/100,000 people per year, with a prevalence of ∼0.24% (3, 4), which is significantly higher in women (5). The etiologies and pathogenesis of RA have been extensively studied, wherein genetic susceptibility (i.e., *HLA DR1*, *TRAF1*, and *STAT4*), epigenetic modification (i.e., DNA methylation, miR146a, and miR155), and environmental factors (i.e., smoking, obesity, periodontitis, and vitamin D deficiency) have been found to promote the loss of immune tolerance, resulting in this disorder (1, 6–8). However, the precise mechanism underlying RA is complex and has not yet been elucidated. Currently, RA treatments, including glucocorticoid, immunosuppressants, and biological agents, are non-specific with an inadequate efficacy, severe adverse reactions, and even life-threatening toxic effects (7, 9).

Mesenchymal stem/stromal cells (MSCs) are a class of stem cells with self-renewal and multipotent properties that are widely available. As such, extensive clinical research has focused on the effects of MSCs in tissue regeneration and protection against injury via the replacement of damaged cells (10, 11). Subsequently, the evidence is increasingly indicating that MSCs play an

immunomodulatory role primarily through the release of extracellular vesicles (EVs) and paracrine factors (e.g., growth factors, hormones, and cytokines) (11, 12). MSCs originate from many types of tissues, including bone marrow (BM), adipose tissue (AT), umbilical cord (UC), cord blood (CB), peripheral blood, dental pulp, liver, and the synovial membrane (12, 13). Generally, MSCs mostly express CD73, CD90, and CD105; however, these surface markers cannot be used to discriminate the source of MSCs. In contrast, MSCs negatively express CD14, CD34, CD45, and HLA-DR. MSCs can escape T cell recognition and exhibit low immunogenicity (14–17).

EVs are a group of lipid-bound vesicles that are released by various cells and play an essential role in the transfer of information between adjacent or distant cells. According to their origin, secretion mechanisms, and properties, EVs are divided into apoptotic bodies, microvesicles (MVs), and exosomes. Apoptotic bodies (50–5,000 nm) are released by dying cells into the extracellular space, and contain intact organelles, chromatin, and small amounts of glycosylated proteins. MVs (100–1,000 nm) originate from plasma membranes. Exosomes (30–150 nm) are formed by the intraluminal buds of multivesicular endosomes (MVEs) (18, 19). Due to the limitations of separation technologies, small EVs (sEVs) (50–200 nm) are commonly used in experimental studies (20). Among the different cells known to produce EVs, MSCs are one of the most prolific cells (21). Phenotypically, MSC-derived sEVs also express the MSC markers CD73, CD90, and CD105, but not CD14, CD34, or CD11b (17). The functions of MSC-EVs are similar to those of MSCs, although the latter are more stable, safe, less toxic, and are able to pass the blood-brain barrier, thus reducing their propensity to trigger immune responses (22–24). MSC-EVs transfer nucleic acids, including DNA, mRNA, and microRNA (miRNA); lipids; proteins; and surface receptors from donor cells to specific recipient cells, thereby protecting signaling molecules from enzymatic degradation during transport. MSC-EVs fuse with the recipient cell membrane either by directly fusing with the plasma membrane, fusing with the endosomal membrane after endocytosis, or by directly binding to the receptor of recipient cells, and then participate in physiological and pathological processes (25–27).

In recent years, studies have shown that MSCs and MSC-EVs may be effective in RA, highlighting their potential immunomodulatory effects. In this review, we aim to discuss recent advances in the use of MSCs and MSC-EVs for the treatment of RA.

IMMUNOMODULATORY EFFECT OF MSCS IN RA

In the past decade, MSC transplantation (MSCT) has been found to be effective in the treatment of RA by reducing joint inflammation, bone erosion, and destruction and alleviating the formation of pannus via immune regulation, anti-inflammation, and differentiation (28, 29). MSCs mainly interact with both innate and adaptive immune cells to modulate immune responses in RA.

MSCs may regulate the proliferation, differentiation, and function of T cells and reduce the production of pro-inflammatory factors. In mouse models with collagen-induced arthritis (CIA), the administration of human AT-derived MSCs (AT-MSCs) inhibited the differentiation of activated CD4+ T cells into T helper (Th) 17 effector cells producing interleukin (IL)-17, but induced the generation of T regulatory cells (Tregs) that secrete IL-10 and negatively regulate the immune response (30). Similar beneficial effects have been reported in RA animal models using various MSC treatments (28, 31, 32). The effects of MSCs on Th17/Treg cell balance have been attributed to various soluble molecules, including indoleamine 2,3-dioxygenase (IDO), IL-10, prostaglandin E2 (PGE2), and nitric oxide (NO), and to the transfer of organelles (32, 33). For example, after co-culturing healthy mice bone marrow-derived MSCs (BM-MSCs) and Th17 from peripheral blood mononuclear cells (PBMCs) of RA patients, the proliferation of Th17 cells and production of IL-17 was inhibited by transferring mitochondria from BM-MSCs to Th17 cells. Simultaneously, mitochondrial transfer from the BM-MSCs of healthy donors was higher than that from the synovium-derived MSCs of RA patients (32). T follicular helper (Tfh) cells, a subset of CD4+ T cells, may help in immunoglobulin affinity maturation and generate live plasma cells and memory B cells (34, 35). Liu et al. found that the number of circulating Tfh cells increased, and was positively correlated with the disease and anti-cyclic citrullinated peptide antibody levels in RA patients (36). Subsequently, they further demonstrated that allogeneic UC-derived MSCs (UC-MSCs) suppressed the proliferation and function of Tfh cells via IDO production, which may be induced by interferon (IFN)-γ *in vivo* and *in vitro*, thereby ameliorating the progression of CIA (37). Endoplasmic reticulum (ER)-stressed MSCs could reduce the number of circulating Tfh cells via higher PGE2 binding with EP2/EP4 and increased IL-6 levels (38).

B cells mainly produce autoantibodies, including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs), but also secret cytokines and act as antigen-presenting cells to promote T cell activation in RA (39). MSCs from healthy donors have been found to suppress B cell proliferation and anti-ACPA and RF production (29, 40). However, the mechanism underlying B cell regulation by MSCs in RA remains unclear. Currently, autologous MSCs injection has been considered to decrease B cell responses by reducing the levels of the B-cell activation factor (BAFF), a proliferation-inducing ligand (APRIL), and BAFF receptors (29). In comparison, in an *in vitro* experiment, BM-MSCs from RA patients co-cultured with B cells from PBMC of healthy donors supported B cell survival, by a mechanism that may not be correlated with BAFF (41). This may be because of the conditional complexity of *in vitro* and *in vivo* experiments. In addition, the inhibition of MSCs on Tfh cells also indirectly affected the proliferation and differentiation of B cells (37).

Dendritic cells (DCs), macrophages, and natural killer (NK) cells are important members of the innate immune response and are regulated by MSCs in various diseases (42–44). However, their interaction with MSCs is scarcely studied in RA. Shin

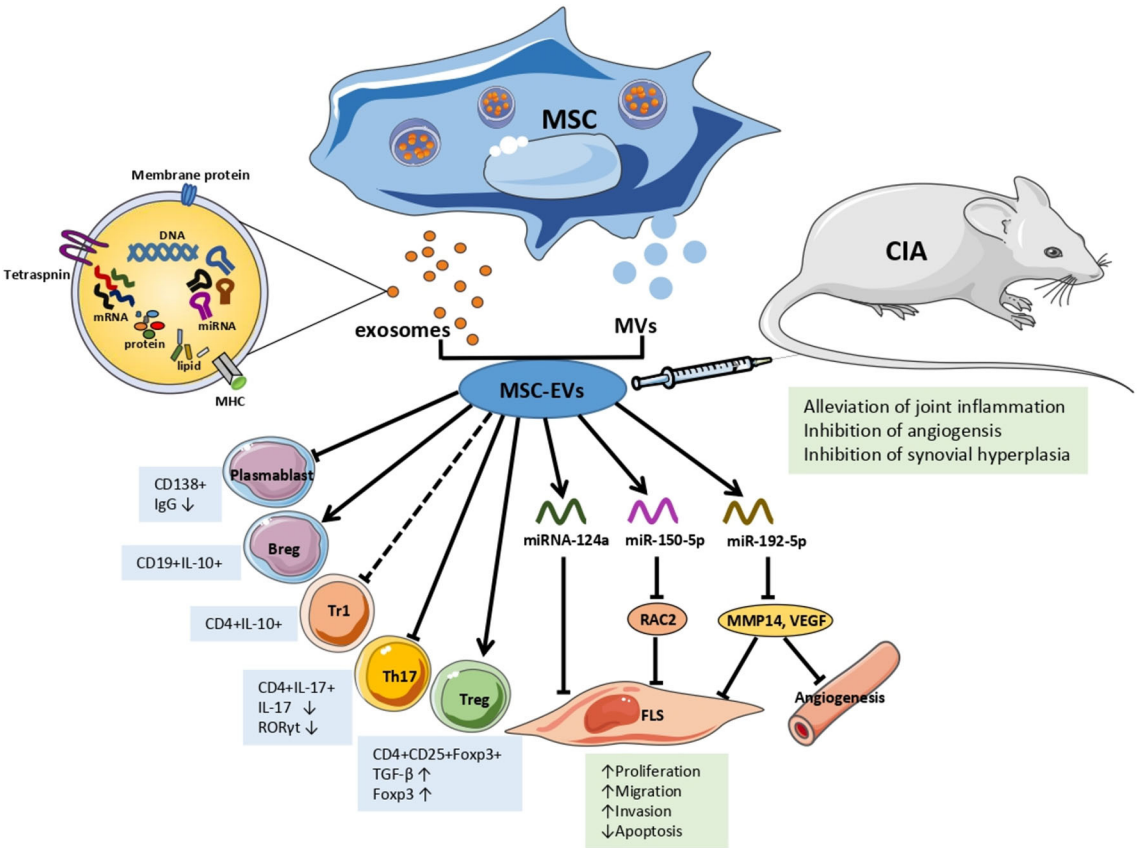

FIGURE 1 | The effects and mechanisms of MSC-EVs in RA. Arrows indicate activation or induction, T-bars indicate inhibition, dotted T-bars indicate inconsistent result, with Tr1 cells increasing *in vitro* but decreasing *in vivo*. MSC, mesenchymal Stem Cell; MVs, microvesicles; EVs, extracellular vesicles; Tr1, T regulatory type 1 cells; Th 17, T helper 17 effector cells; Treg, T regulatory cells; FLS, fibroblast-like synoviocytes; IgG, immunoglobulin G; TGF-β, transforming growth factor β; IL, interleukin; miRNA/miR, microRNA; RAC2, ras-related C3 botulinum toxin substrate 2; MMP14, matrix metalloproteinase 14; VEGF, vascular endothelial growth factor; CIA, collagen-induced arthritis mice model.

et al. demonstrated that MSCs inhibited the activation of M1-type macrophages and induced the generation of M2-type macrophages via the tumor necrosis factor (TNF)-α-mediated activation of cyclooxygenase-2 (COX-2) and TNF-stimulated gene-6. This was accompanied by the negative regulation of the nucleotide-binding domain, leucine-rich repeat pyrin 3 (NLRP3) inflammasome-mediated IL-1β secretion, and caspase-1 production in macrophages through an IL-1β feedback loop (45). In addition, MSCs from systemic juvenile idiopathic arthritis patients were found to inhibit the differentiation of monocytes to DCs and suppress NK cell activation (46). Li et al. found that the combination of tolerogenic DCs and MSCs had a synergistic immunosuppressive effect on CIA mice by polarizing Th cells and inhibiting pro-inflammatory cytokines (47).

CLINICAL MSC TRIALS IN RA

In recent years, clinical research on the use of MSC therapy for the treatment of RA has increased. The first randomized clinical trial (RCT) using allogeneic expanded AT-MSCs (Cx611) for RA treatment was conducted in 2011 as a multicenter, single blind, and placebo-controlled phase Ib/IIa clinical trial. A total of 53 refractory RA patients were enrolled and assigned to three cohorts with different doses (1, 2, or 4 million cells/kg) and a placebo cohort, to evaluate the safety and tolerability of Cx611. The results indicated that the infusion of Cx611 was generally well-tolerated. One patient with dose-limiting toxicity (DLT) presented lacunar infarction. Most adverse events (AE) were mild or of moderate intensity. Although the most common symptoms were fever and infection, it was difficult to discern whether these were symptoms or simply side effects of Cx611 (48). In a phase Ia RCT investigating the efficacy and safety of the intravenous infusion of human CB-derived MSCs (hCB-MSCs), 9 RA patients were divided equally among three groups, each receiving a single intravenous infusion of hCB-MSCs at different dosages. No short-term AE or DLT were reported 4 weeks after infusion. Moreover, the DAS 28 (28-joint disease activity score) was significantly decreased, pro-inflammatory cytokines were reduced, and IL-10 levels were increased 24 h after infusion (49). Similarly, a single-center RCT selected 30 RA

patients with knee involvement to receive either intra-articular knee autologous BM-MSCT ($n = 15$) or normal saline ($n = 15$). Following the transplantation of 40 million autologous BM-MSCs, although no statistically significant results between the two groups were noted in the majority of the outcome measures, favorable effects on joint inflammation symptoms were observed, with an improved standing time in the MSCT group ($p = 0.02$). Moreover, MSCT treatment helped to reduce the dosage of MTX and prednisolone during the initial 6 months of follow-up, although not after 1 year. Importantly, no AEs were observed after MSC administration or during follow-up (50). Similarly, a clinical trial in Iran investigated whether injections of autologous BM-MSCs relieved the symptoms of refractory RA patients (51). These clinical trials found that MSC therapy for RA, especially refractory RA, is safe, well tolerable, and effective.

IMMUNOMODULATORY EFFECTS OF MSC-EVS IN RA

Recent studies have indicated that the mechanisms underlying the interaction between MSC-EVs and recipient cells are not unique in terms of their physiological or pathological processes in RA (**Figure 1**). Ma et al. found that both human UC-MSCs and the EVs secreted by them inhibited the proliferation of T cells, promoted T cell apoptosis, decreased RORγ levels, increased Foxp3 levels, and regulated the balance of Treg/Th17 cells in *in vitro* and *in vivo* experiments, resulting in delayed radiological progression and synovial hyperplasia inhibition (52). Notably, the partial effect of MSC-EVs was different from that of parental MSCs in RA. MSC-exosomes increased the number of Treg, whereas MSCs did not. And MSCs were more capable of reducing the number of CD4+IFN-γ+ T lymphocytes. Compared with parental BM-MSCs and MVs, the exosomes increased the number of Treg cells. In addition, this study showed that MSC-exosomes inhibited plasmablasts but generated Breg cells (53).

Based on the fact that EVs are able to transfer information to recipient cells, subsequent studies characterized the mechanisms by which EVs, particularly through miRNAs, are involved in RA. Chen et al. were the first to report that BM-MSC-EVs transferred miR-150-5p to the joint cavity. Compared with osteoarthritis patients, the expression levels of miR-150-5p in the serum, synovial tissues, and fibroblast-like synoviocytes (FLS) of RA patients were significantly decreased, whereas the expression levels of matrix metalloproteinase (MMP) 14 and vascular endothelial growth factor (VEGF) were increased. MiR-150-5p was effectively transfected into BM-MSCs *in vitro* and transferred by exosomes to RA-FLS. MSC-exosomal miR-150-5p suppressed the expression of the target genes MMP14 and VEGF by directly binding to their 3'-UTRs, thereby reversing the migration and invasion of RA-FLS and HUVEC tube formation induced by pro-inflammatory factors, including IL-1β, transforming growth factor β (TGF-β), and TNF-α. *In vivo*, the effect of MSC-exosome-miR-150-5p injection was consistent with those mentioned above, wherein MSC-exosome-miR-150-5p inhibited angiogenesis and alleviated joint inflammation (54). Recently, miR-192-5p expression was found to be decreased in human RA-FLS, wherein a dual luciferase reporter gene assay showed that miR-192-5p directly targeted and negatively regulated ras-related C3 botulinum toxin substrate 2 (RAC2). In a CIA rat model, MSC-exosomal miR192-5p was transferred to the synovial tissue via the blood circulation after injection, and significantly reduced the levels of RAC2, decreased the clinical score, and suppressed synovial hyperplasia and joint destruction compared with rats injected with BM-MSCs-exosome-NC. Additionally, MSC-exosome-miR-192-5p inhibited the levels of pro-inflammatory cytokines, including PGE2, IL-1β, and TNF-α, in synovial tissues and serum, and reduced the release of NO and inducible NO synthase (iNOS) in the sera of CIA rats (55). Another *in vitro* experiment found that the exosome number and miRNA-124a levels increased in MH7A cells (RA-FLS cell lines) after co-culturing MH7A with human MSC-EV. With miRNA-124a (hMSC-124a-EV) overexpression, the proliferation of MH7A was inhibited by hMSC-124a-EV and hMSC-EV compared with that in the control group. However, the cells were blocked in the G0/G1 and S phases, respectively. The invasion and migration of MH7A were also suppressed, while apoptosis was promoted. Moreover, the effect of hMSC-124a-EV treatment was more marked than that of hMSC-EV (56).

PROSPECTS AND CHALLENGES IN THE CLINICAL APPLICATION OF MSCS AND MSC-EVS IN RA

With an increasing number of studies, MSCs have been found to play an immunomodulatory role in numerous autoimmune diseases through the production of soluble factors, and the transfer of EVs containing messaging molecules (11, 57–59). In addition to immune regulation, MSCs can induce osteogenic and chondrogenic differentiation, and regulate inflammatory factors, highlighting it as a promising therapy for RA. Currently, most clinical trials of MSCT therapy for RA have focused on refractory RA patients who have not responded to traditional disease modifying antirheumatic drug (DMARDs) therapy, without any serious AEs associated with MSCT treatment. However, the use of MSCs in therapeutic treatments still faces many challenges. Several studies have found that MSCs are associated with carcinogenic risk when injected in animal models (60–63). Allogeneic MSCs have an immunosuppressive effect on tumor cells, allowing them to evade detection and destruction by the adaptive immune regulatory system via the action of CD8+ T cells, leading to the growth of allogeneic tumor cells (61). MSCs could also secrete VEGF to induce angiogenesis (62), contributing to tumor stroma formation, and favor tumor cell proliferation, invasion, and migration (60). The immunosuppressive effects of MSCs in CIA are also debatable. While MSCs can inhibit anti-CD3-induced T-cell proliferation *in vitro*, they do not affect T cell proliferation nor the development of CIA (64). Factors including the type of MSCs, culture conditions, treatment time, number of injected cells, injection route, and treatment regimen can lead to different results. A recent study compared the effects of three different types of

MSCs infused into CIA mice and found that the most effective treatment was UC-MSCs, followed by BM-MSCs (65). The efficacy of allogeneic and autologous MSCs remains debatable, Rozier suggests that autologous MSCs may be involved in the physiopathology of systemic sclerosis (66). Therefore, RCT are necessary to compare the efficacy and safety of autologous and allogeneic MSC therapy in RA. BM-MSCs from RA patients were also found to promote Th17 cell activation and expansion via caspase 1 activation (67). In addition, different conditions also influence the effect of MSCs. For example, epigenetically-modified MSCs (combination of hypomethylating agents and histone deacetylase inhibitors) have a high immunoregulatory effect in RA (68). Consequently, determining how long the immunomodulatory effects of MSCs last will need to be solved in clinical practice, the results of which could provide a theoretical basis and support for their use in the treatment of RA.

Compared with MSC treatment, which may cause abnormal differentiation and tumor formation, MSC-EVs are more effective, stable, and safer in alleviating inflammation of CIA, with broader prospects. EVs carry numerous DNAs, RNAs, proteins, and lipids from MSCs and transfer them to the recipient cells. Due to several advantages, including the ability to pass the blood-brain barrier and their low immunogenicity, EVs are natural carriers for drugs and exogenous nucleic acids, which can be loaded in donor cells before being released into the extracellular environment (69, 70). More importantly, using EVs to transfer miRNAs can prevent these from being degraded, allowing miRNAs to negatively regulate target protein expression at the post-transcriptional level. In addition to their treatment potential, several studies have reported that miRNAs secreted by MSC-EVs (MSC-EV-miRNAs) regulate diverse signaling pathways by targeting specific proteins, thereby influencing the development of RA. Therefore, MSC-EV-miRNAs are potential biomarkers for use in novel cell-free therapeutic strategies for RA.

Although MSC-EVs have been used in preclinical RA studies, several issues still remain unsolved. Firstly, when MSCs from different tissues are in distinct differentiation states, the content and types of molecules assembled by EVs may be different, thereby affecting their function at recipient cells and causing changes to physiological processes. MSC-EV miRNAs do not randomly enter EVs, however, the sorting mechanism by which cells are adjusted and selected from maternal cells is unknown. Generally, hundreds of differentially expressed miRNAs could be found in MSC-EVs by sequencing or microarray assays, however, no studies have performed miRNA expression profiling on MSC-EVs between RA and healthy individuals. Currently, dozens of miRNAs have been reported to affect the proliferation and function of FLS, previous studies also focused on the effect of MSC-EV-miRNAs on FLS, further studies on other cells are also needed. Additionally, whether the complex regulatory network of miRNAs and their target genes may trigger other diseases remains unclear and needs further study. Secondly, MSC-EVs secrete many other signaling molecules. Eirin et al. (71) integrated transcriptomic and proteomic analyses and found that the proteins, transcription factors, and translational regulators derived from MSC-EVs are involved in the mechanism of tissue repair in the recipient cell. Further studies on the interactions of the molecules that affect RA are necessary. Thirdly, EVs are separated in different ways, without standards, and in a time-consuming manner. Although commercial exosome extractants are currently being used, they contain non-exosome contaminants, such as lipoproteins, which need to be purified. Lastly, the findings presented here will need to be replicated on a large scale in clinical trials to assess the safety, effectiveness, and persistence of MSC-EVs in RA patients.

AUTHOR CONTRIBUTIONS

HL, RL, TL, and LY wrote sections of the manuscript. QX and GY critically revised the manuscript. QX reviewed and approved the version to be published. All authors contributed to manuscript revision, read, and approved the submitted version.

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Molecular and Functional Diversity of Distinct Subpopulations of the Stressed Insulin-Secreting Cell's Vesiculome

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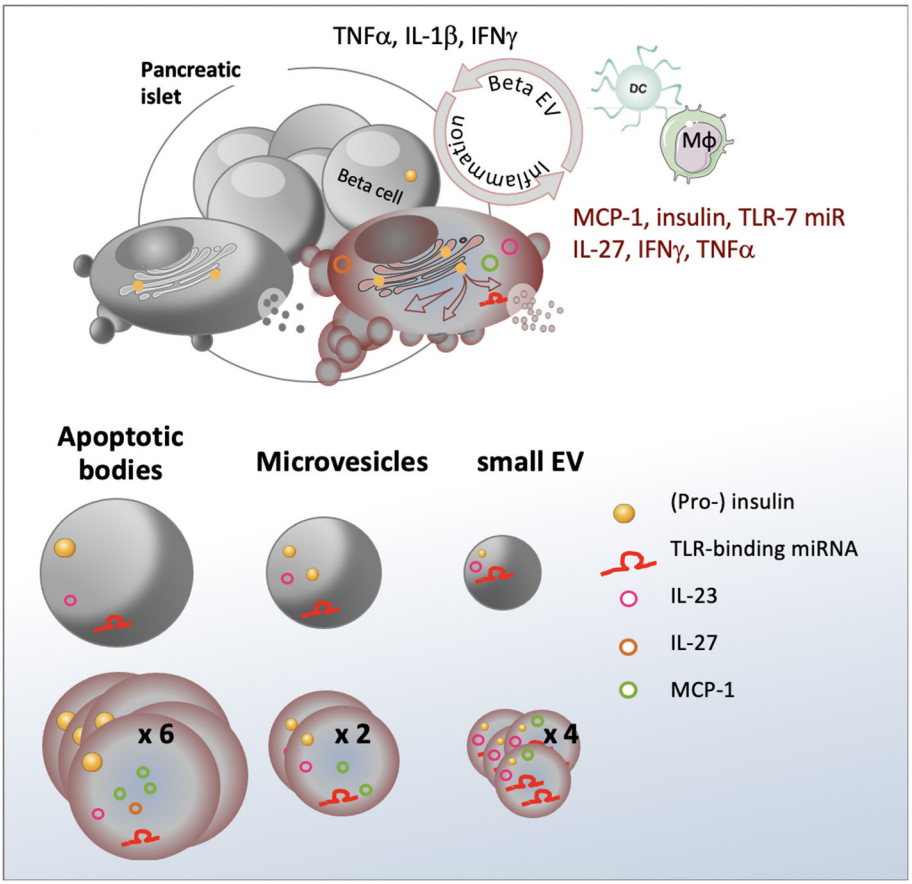
Beta cell failure and apoptosis following islet inflammation have been associated with autoimmune type 1 diabetes pathogenesis. As conveyors of biological active material, extracellular vesicles (EV) act as mediators in communication with immune effectors fostering the idea that EV from inflamed beta cells may contribute to autoimmunity. Evidence accumulates that beta exosomes promote diabetogenic responses, but relative contributions of larger vesicles as well as variations in the composition of the beta cell's vesiculome due to environmental changes have not been explored yet. Here, we made side-by-side comparisons of the phenotype and function of apoptotic bodies (AB), microvesicles (MV) and small EV (sEV) isolated from an equal amount of MIN6 beta cells exposed to inflammatory, hypoxic or genotoxic stressors. Under normal conditions, large vesicles represent 93% of the volume, but only 2% of the number of the vesicles. Our data reveal a consistently higher release of AB and sEV and to a lesser extent of MV, exclusively under inflammatory conditions commensurate with a 4-fold increase in the total volume of the vesiculome and enhanced export of immune-stimulatory material including the autoantigen insulin, microRNA, and cytokines. Whilst inflammation does not change the concentration of insulin inside the EV, specific Toll-like receptor-binding microRNA sequences preferentially partition into sEV. Exposure to inflammatory stress engenders drastic increases in the expression of monocyte chemoattractant protein 1 in all EV and of interleukin-27 solely in AB suggesting selective sorting toward EV subspecies. Functional *in vitro* assays in mouse dendritic cells and macrophages reveal further differences in the aptitude of EV to modulate expression of cytokines and maturation markers. These findings highlight the different quantitative and qualitative imprints of environmental changes in subpopulations of beta EV that may contribute to the spread of inflammation and sustained immune cell recruitment at the inception of the (auto-) immune response.

Keywords: type 1 diabetes, extracellular vesicles, apoptotic bodies, microvesicles, exosomes, microRNA, Toll-like receptor



GRAPHICAL ABSTRACT | Inflammation stimulates release of a heterogeneous population of beta EV with differential expression of immunogenic substances involved in immune cell recruitment and activation.

HIGHLIGHTS

- Stress engenders an up to four-fold increase in the volume of the vesiculome and enhanced auto-antigen release
- Cytokines are selectively sorted into EV subspecies
- TLR-binding microRNAs are enriched in sEV
- EV from stressed beta cells promote dendritic and macrophage cell activation.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease caused by the destruction of the insulin-producing beta cells in the pancreas leading to chronic hyperglycaemia and serious long-term complications such as cardiovascular disease, neuropathy, nephropathy and blindness [reviewed in (1)]. More than 30 million of people suffer from T1D worldwide (www.idf.org). T1D and its sequelae reduce life expectancy of patients by more than eleven years (2). Pathogenesis of T1D is characterized by inflammatory events in the beta cell microenvironment causing innate immune activation followed by progressive infiltration of the islets of Langerhans in the endocrine pancreas by auto-reactive cytotoxic T-lymphocytes. Disease etiology has only partially been elucidated, but results from a complex interplay between genetic and environmental factors collectively engendering functional defects in the immune system and the beta cell itself. Environmental changes in toxins, pathogens, nutrients in particular glucose overload, and low physical activity have been suggested to be responsible for the 3.4% annual increase in disease incidence (3). Due to its demanding secretory function, the beta cell is extremely sensitive to stress. Insulin accounts for up to half of the cell's protein content (4) and rapid changes can exceed the endoplasmic reticulum's (ER) folding capacities leading to the accumulation of misfolded proteins e.g. potential neoantigens within the lumen of the ER. By interaction with built-in sensors, these misfolded proteins trigger the unfolded protein response (UPR), a signaling pathway that aims to restore homeostasis by enhancing the cell's folding capacity and translational attenuation. However, chronic stress can cause the UPR to initiate apoptosis. Beta cell stress and apoptosis has been associated with T1D pathogenesis (5, 6), yet, how stressed beta cells trigger innate immune responses at disease initiation has not been fully elucidated.

Extracellular vesicles (EV) are membrane-bound vesicles released by healthy and diseased cells. Three major types of

EV can be distinguished based on their origin and biogenesis pathway: apoptotic bodies (AB), microvesicles (MV) and exosomes [reviewed in (7)]. AB are large 1,000 - 5,000 nm vesicles released by cells undergoing apoptosis (8). In contrast to other EV, AB may contain cellular organelles and their constituents including elements from the nucleus, mitochondria, the Golgi apparatus, the ER and the cytoskeleton. MV are formed by outward budding and scission of the plasma membrane. Typically, the size of MV ranges from 100-1,000 nm. In line with their pathway of formation, MV contain mainly cytosolic and plasma membrane-associated proteins such as tetraspanins. Exosomes result from the inward budding of the membrane of the endosome leading to the formation of 30-120 nm intraluminal vesicles that can be released upon fusion of the endosome with the plasma membrane. Throughout its maturation, the endosome is an important site of bi-directional translocation of substances between the cytoplasm and the endosome. In consequence, the packing of specific cargo molecules into EV and their release are intimately linked to the state of the releasing cells. As conveyors of biological active material from their cell of origin to neighboring or distant recipient cells, EV act as mediators in cell-to-cell communication fostering the idea that they may constitute the missing link between beta cell stress and immune activation [reviewed in (9)].

While beta AB have been successfully used to induce tolerance in diabetes-prone non obese diabetic (NOD) mice (10), evidence accumulates that exosomes derived from pancreatic beta cells contribute to T1D development. Strikingly, all known beta auto-antigens are directly or indirectly linked to secretory pathways and 13 localize to secretory granules, synaptic vesicles, the ER and the trans-Golgi network (TGN) (11). Several studies showed that human and mouse beta EV contain major auto-antigens of type 1 diabetes such as glutamic acid decarboxylase 65 (GAD65), glucose-transporter 2 (GLUT-2), islet-associated antigen 2 (IA-2), zinc transporter 8 (Znt8), and insulin (12–15). Interconnections between the TGN secretory pathway of autoantigens and the endosomal compartment where exosome biogenesis occurs have convincingly been demonstrated by immunofluorescent studies co-localizing the auto-antigen GAD65 with the TGN protein 38, but also with endosomal ras-related protein in brain 11 (Rab11) and the exosomal markers flotillin-1 (FLOT1) and CD81 in vesicular structures at the peripheral membrane (12). Exosomes from healthy beta cells efficiently trigger antigen-presenting cell (APC) activation and T-cell proliferation *in vitro* and accelerate islet infiltration by immune cells in non-obese diabetic resistant mice *in vivo* (13). In human T1D patients, healthy beta EV mediate B- and T-cell activation (16). It has further been hypothesized that aberrant sorting in stressed beta cells could fuel release of misfolded immunogenic proteins and danger-associated molecular patterns (DAMP) inside EV. With the aim to explore roles of beta EV in T1D pathogenesis, attempts are made to recreate the beta cell environment by adding a mild cocktail of the proinflammatory cytokines TNFα, IFNγ, and IL-1β present in the pancreas at disease initiation (12, 17, 18). EV ferry short non-coding microRNA (miRNA) that have the aptitude to repress translation of target genes in recipient cells (19), a well-documented mechanism termed RNA interference (RNAi) (20). MiRNA in exosomes derived from beta cells under inflammatory conditions contribute to the spread of beta cell apoptosis (17). However, the biological relevance of miRNA transfer has been questioned by estimates of 1,000 copies required per recipient cells to allow for effective target gene regulation (21). More recently, six specific GU-rich miRNA sequences have been identified (let-7b/c, miR-21, miR-7a, miR-29a/b) that may stimulate immune signaling by binding to the Toll-like receptor-7 (TLR-7), independently of RNAi. Packed into EV, these miRNA sequences act as DAMP exacerbating inflammation in cancer, neurological and autoimmune settings (22–28). Beta EV T- and B-cell activation in NOD mice was impaired in NOD. *MyD88*$^{-/-}$ mice suggesting a role for TLR-signaling in EV-mediated immune responses (13, 29).

To date, the molecular and functional diversity of EV in the beta cell's secretome has not been thoroughly explored. The majority of beta EV studies focuses on small exosome-like vesicles and AB and no studies on contributions of beta MV have been published to our knowledge. Because subtypes of beta EV potentially exert detrimental or protective effects in the immune balance, side-by-side comparisons are mandatory to evaluate their role in T1D pathogenesis. We herein sought to investigate on changes in the relative composition of the vesiculome as well as the partition of the candidate autoantigen insulin and immunostimulatory miRNA sequences inside AB, MV and exosome subpopulations derived from equal amounts of healthy and stressed beta cells and their impact on innate immune responses. As current isolation methods do not allow distinguishing between exosomes of endosomal origin and small MV, the latter will be called small EV (sEV) throughout this study.

MATERIALS AND METHODS

Mice

NOD/ShiLtJ mice were obtained from Charles River Laboratories (L'Abresle, France), bred and housed in a pathogen-free environment at ONIRIS' Rodent Facility (Agreement #44266). Six to ten weeks old female mice were used in the study. All animal procedures were approved by the Pays de la Loire regional committee on ethics of animal experiments (APAFIS#9871). All possible efforts were made to minimize animal suffering.

Cell Culture

MIN6 cells (kindly provided by Prof. Jun-ichi Miyazaki, University Medical School, Osaka, Japan) were cultured at a density of 1.5×10^5 cells/cm^2 in DMEM high glucose medium (Life Technologies, Saint Aubin, France) supplemented with 10% FCS (Eurobio, Les Ulis, France) and 20 μM beta-mercaptoethanol (SIGMA, Saint Quentin Fallavier, France) (30). Cell cultures were regularly assessed for mycoplasma contamination using the mycoplasma quick test (Lonza, Basel, Switzerland). For EV production, MIN6 cells were plated at a density of 3×10^5 cells/cm^2 in DMEM medium. The following day, the medium was replaced by OptiMEM (Life Technologies) supplemented with 1% exosome-precleared FCS (FCS^{exo-}) obtained through overnight centrifugation at 120,000 x g on a SW41 Ti swinging bucket rotor on a L7-55 centrifuge

(Beckman Coulter, Villepinte, France) in polyallomer tubes (Beckman Coulter). For experimental induction of cellular stress, MIN6 cells were either exposed to pro-inflammatory cytokines (17U/mL IL-1β, 167U/mL TNFα and 17U/mL IFNγ; all cytokines were supplied by eBioscience Affymetrix, Paris, France) 254 nm UVB irradiation 10 mJ, BLX-254, Vilber Lourmat, Marne la Vallee, France), low oxygen tension (1% O_2) or left untreated. Supernatants containing MIN6 exosomes were harvested 30 h later. At harvest, cell viability of untreated cells was ≥90% in compliance with guidelines to minimize apoptotic EV input in vesicle preparations (31, 32).

RAW264.7 cells (ATCC #TIB-71) were maintained in RPMI 1640 medium (Life Technologies), supplemented with 10% FCS and 2 mM L-glutamine (Eurobio). Cells were split twice per week by gently scrapping of the cells in cold PBS 2 mM EDTA solution. For immune assays, 5×10^4 RAW264.7 cells were plated on flat-bottomed 96-well plates in RPMI 10% FCS^{exo-} medium 2 mM glutamine.

Bone marrow-derived dendritic cells (bmDC). Bone marrow progenitor cells were isolated from femurs and tibias from $NOD_{Shi/LTJ}$ female mice and cultured in complete RPMI medium (Eurobio) i.e., supplemented with 1% heat-denatured syngeneic mouse serum along with 1 mM sodium pyruvate, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, non-essential amino acids and 20 µM beta-mercaptoethanol. Medium was supplemented with 20 ng/mL GM-CSF (PeproTech, Neuilly-sur-Seine, France) and 5 ng/mL IL4 (BioLegend, London, UK) and 2 x 10^6 cells were cultured in 10 mL of medium per 100 mm Petri dish for 10 days. On day four and nine, an additional 10 and 5 mL of complete culture media were added, respectively. On day 7, 10 mL of culture media were refreshed. On day ten, flow cytometry routinely revealed $CD11c^+ > 90\%$ purity of bmDC cultures.

For immune assays, 0.3×10^6 bmDC were cultured in 48-well plates coated polyhydroxyethylmethacrylate (Sigma) for 18 h in RPMI 1% exosome-depleted mouse serum (MS^{exo-}) supplemented with additives as described before. EV or Toll-like receptor–ligands (TLR-L) (InvivoGen, Toulouse, France): imiquimod (IMIQ; TLR-7L), resiquimod (R848; TLR-7/8L) and polyinosine-polycytidylic acid (PIC; TLR-3L) were used at concentrations as indicated on figures.

Antibodies & Reagents

Phenotypic analysis was performed by flow cytometry on a FACS Aria (BD Biosciences, Le Pont de Claix, France) or MACSQuant (Miltenyi, Paris, France) instrument using the following antibodies: CD11c (N418, BioLegend, Toulouse, France), CD86 (GL-1, BioLegend), major histocompatibility complex II (MHC II; 103.6.2, BD Biosciences), CD40 (3/23, BioLegend), CD115 (CSF-1R, BioLegend), CD11b (M1/70, BioLegend), B220 (RA3-6B2, BD Biosciences). Zombie-NIR (BioLegend), DAPI (Fisher Scientific, Illkirch, France) or Viobility 405/520 (Miltenyi) dyes were used to discriminate death cells. Results were analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA) or Flowlogic (Miltenyi) software.

Cytokine secretion into cell culture supernatants was quantified by ELISA using IFNa, TNFα (R&D systems) according to the suppliers' protocols. Cytometric bead assay analysis of IL-23, IL-1β, IFNγ, TNFα, MCP-1, IL-12p70, IL-1β, IL-10, IL-6, IL-27, IL-17A, IFNγ, and GM-CSF was performed using a predefined 13-plex Mouse Inflammation panel (BioLegend). To assess for internal as well as surface-associated cytokines, 30 min of incubation in 0.5% Triton X-100 and 1 min. of sonication were performed prior to CBA of cytokine expression in EV. Total mouse insulin in MIN6 EV was quantified by ELISA (Mercodia, Upsala, Sweden).

Protein concentration was determined by a Bradford protein assay using Coomassie plus assay reagent (Fisher Scientific). Optical densities were read on Fluostar (BMG LABTECH, Champigny sur Marne, France) and Nanodrop2000 (Fisher Scientific) spectrophotometers following the supplier's recommendations.

Markers of Hypoxia

For detection of the endogenous marker of hypoxia HIF-1α, MIN6 cells were cultured as indicated for 30 h in normoxia or hypoxia. As soon as the hypoxia chamber was opened, cells were washed and lysed with 1 mL of 50 mM Tris (pH 7.4), 300 mM NaCl, 10% (w/v) glycerol, 3 mM EDTA, 1 mM MgCl2, 20 mM b-glycerophosphate, 25 mM NaF, 1% Triton X-100, 25 µg/mL Leupeptin, 25 µg/mL Pepstatin and 3 µg/mL Aprotinin lysis buffer for 30 min on a rotating platform at 4°C. The cells were centrifuged at 2,000 × g for 5 min and supernatants were stored at −80°C. Proteins were quantified using the Bradford assay and HIF-1α was measured by ELISA following the supplier's instructions (R&Dsystem). Optical Density at 450 nm was measured using the FLUOstar Optima Microplate Reader (BMG Labtech, Champigny sur Marne, France).

The exogenous marker pimonidazole forms stable adducts with thiol groups in cellular proteins under hypoxia. Briefly, 3×10^5 cells/cm^2 MIN6 were cultured for 24 h in DMEM culture medium. After washing, cells were incubated with 100 µM pimonidazole (Hypoxyprobe-1 plus; Hypoxyprobe Inc, Burlington, USA) in OptiMEM 1% FCS^{exo-} for 2 h in normoxia or hypoxia. The cells were fixed with 4% PFA, permeabilized with PBS 0.1% Triton 4% FCS followed by saturation in PBS 5% rabbit serum for 1 h. For detection of pimonidazole adducts, cells were incubated overnight at 4°C with FITC-conjugated mouse anti-pimonidazole monoclonal antibody (1:100). The cells were washed and imaged on a fluorescent microscope (AXIO Zeiss, Leica, Nanterre, France).

Caspase Assay

Apoptosis was assayed by fluorescent caspase-3/7 substrate cleavage staining. Briefly, 3×10^5 cells/cm^2 MIN6 cells were cultured in eight-chamber labteks with coverslips. After overnight culture, cells were switched to OptiMEM 1% FCS production medium and exposed to cytokines, UV irradiation, hypoxia or left untreated. Eighteen hours later, cells were treated with 2 mM caspase-3/7 detection reagent (Fisher Scientific) for 30 min. at 37°C and counterstained with 1 µg/ml Hoechst 33342 (Sigma). Cells were fixed with 4% PFA, washed with PBS and overlaid with Mowiol (Sigma) before analysis by fluorescence confocal imaging on a LSM780 confocal microscope (Zeiss,

Oberkochen, Germany). Tiles of nine images per well were acquired and processed for semi-automatic quantitative analysis of caspase-positive cells using an in-house macro and Fiji software. Total cell count was set equal to the number of Hoechst positive regions.

Separation of Beta-EV subpopulations

EV were collected from MIN6 supernatants using a method combining differential centrifugation, ultrafiltration and size-exclusion chromatography steps. Briefly, 90 ml of 30-h supernatants from MIN6 cells were centrifuged immediately after harvest at 300 x g 10 min, 2,000 × g, 20 min (AB) and 16,600 × g, 20 min (MV). The pellets containing AB and MV were washed with PBS or RPMI and centrifuged again before use. The 16,500 supernatants were filtered 0.2 μm and concentrated on an AMICON MWCO-100 kDa cellulose ultrafiltration unit (Dutscher, Issy-les-Moulineaux, France). Approximately, 100 μl of concentrates were recovered and passed through a size exclusion chromatography column (IZON, Lyon, France). sEV were collected following the supplier's recommendations in flow-through fractions four and eight for qEV single and qEV original, respectively. EV were stored at 4°C for 1–3 days or at −80°C for up to 1 year. All assays of biological activity were carried out using fresh EV. For transcriptomic analyses, 90 ml of 30-hours supernatants from MIN6 cells were centrifuged immediately after harvest at 300 x *g* 10 min., followed by centrifugation at 16,500 x g, 20 minutes to collect large EV (LEV) comprising both AB and MV.

Tunable Resistive Pulse Sensing (TRPS)

The size and concentration of EV were analyzed by the TRPS technique using a qNANO instrument and NP2000 (AB), NP800 (MV) and NP100 (sEV) nanopores (IZON, Lyon, France). All samples were diluted in PBS 0.03% Tween-20. After instrument calibration using 110 nm, 710 nm or 2,000 nm calibration beads (Izon), all samples were recorded with at least two different pressures. Respective particle volumes $V=4/3\pi r^3$ x nb particles were calculated based on the mean particle diameter measured, assuming spherical shape.

Immunoblotting

Protein lysats of cells, AB and MV were prepared in RIPA buffer containing a cocktail of protease inhibitors (Sigma). Proteins were denatured in Laemmli buffer and separated by 4-12% gradient SDS-PAGE in non-reducing (tetraspanins CD81, CD63, CD9) or reducing (all other) conditions and transferred to a nitrocellulose membrane (CD63, flotillin-1, peIF2a and CHOP; Fisher Scientific) or PVDF membrane (all other; BIO-RAD, Marnes La Coquette, France). Membranes were incubated with primary antibodies CD63 (NVG-2; 1:1000; BioLegend), CD81 (Eat-2; 1:1000; BioLegend), CD9 (EM-04; 1:1000; Abcam, Cambridge, UK), polyclonal rabbit anti-calnexin antibody (1:1000; Euromedex, Souffelweyersheim, France), β–actin (W16197A; 1:20,000; Biolegend), flotillin-1 (W16108A; 1:1000; Biolegend), peIF2a (D9G8; 1:1000; Ozyme) and CHOP (D46F1; 1:1000; Ozyme) blocked by either TBS 0.05% Tween-20 4% BSA (CD81, peIF2a, CHOP) or TBS 0.05% Tween-20 5% milk (CD9, CD63, calnexin, flotillin-1), followed by incubation with cognate HRP-conjugated secondary antibodies 1:100,000. The signals were detected with enhanced chemiluminescence substrate (ECL West Pico Femto, Fischer Scientific) on a Fusion FX6 instrument (Fisher Scientific).

Cryo-Electron Microscopy

MV and sEV were applied onto glow-discharged perforated grids (C-flat^{TM}), prepared using an EM-GP (Leica, Germany) at room temperature in a humidity saturated atmosphere. EV samples were mixed with 10 nm diameter gold nanoparticles at a concentration of 80nM (33) and four μl of the mixture were deposited on the grids. Excess sample was removed by blotting for 0.8 to 1.2 seconds before snap-freezing of samples into liquid ethane and storage in liquid nitrogen until observation. The grids were mounted in a single-axis cryo-holder (model 626, Gatan, USA) and the data were collected on a Tecnai G^2T20 sphera electron microscope (FEI company, The Netherlands) equipped with a CCD camera (US4000, Gatan) at 200 kV. Images were taken at a nominal magnification x 29,000 in low-electron dose conditions. For cryo-electron tomography, single-axis tilt series, typically in the angular range ±60°, were acquired under low electron doses (∼0.3 $e^-/Å^2$) using the camera in binning mode 2 and at nominal magnifications of 25,000x and 29,000x, corresponding to calibrated pixel sizes of 0.95 and 0.79 nm at the specimen level, respectively. Tomograms were reconstructed using the graphical user interface eTomo from the IMOD software package (34, 35).

Fluorescence Imaging of EV

AB and MV were separated from MIN6 cell supernatant and stained with MemBright (MB) dye (MB-Cy3 and MB-Cy5 (200 nM) kindly provided by M Collot). The mixture was incubated for 30 minutes at room temperature with gentle rotation. EV were then centrifuged at 2,000 x g (AB) or 16,600 x g (MV) for 20 minutes and washed in PBS. Samples were transferred into Labtek wells and overlaid with Mowiol (Sigma) before acquisition of images in superresolution mode Airyscan on a Zeiss LSM780 instrument.

Quantitative RT-PCR Analysis

Total RNA including miRNA was extracted from MIN6 cells or EV derived from an equal amount of cells using the miRVana kit (Fisher Scientific) or TriReagent (SIGMA), respectively. During the initial lysis step, all samples were spiked with 10^{10} copies of a synthetic analog of ath-miR-159 (Eurogentec, Angers, France). Following reverse transcription using RT-stem-loop primers, extravesicular cDNA was pre-amplified for all miRNA except the spike ath-miR-159 by 10-14 cycles of PCR using Taqman probe reagent (Solisbiodyne, Tartu, Estonia) and Taqman assays (Fisher Scientific), followed by 40 cycles of PCR on an ABI7300 instrument (Fisher Scientific). For each target, standard curves were generated using serial sample dilutions. Relative quantities (in arbitrary units) of miRNA in samples were inferred by the relative standard curve method and normalized with respect to the spike and untreated controls.

Statistical Analysis

Statistical tests were performed using either Prism GraphPad Software (Comparex, Issy-les-Moulineaux, France) or R 3.6.0 (36) with RStudio (37) and lsmeans (38) and lme4 (39) packages using tests as indicated in the figure legends. Confidence levels of 95% were considered significant. For linear mixed model, the parameters were the "type of EV" or the "treatment of the producing cells". No interaction test was deemed necessary, as analysis was performed for all "treatments" of a single "type of EV" or vice-versa. The random parameter was the individual "experiment". *Post-hoc* analysis was performed by the Tukey's range test for pairwise comparisons on calculated least-square means.

RESULTS

Primary islet inflammatory events have been associated with beta cell stress and failure at the origin of T1D pathogenesis (40, 41). With the aim to study the impact of cellular stress on the beta vesicular secretome, murine MIN6 beta cells were either left untreated (CTL) or exposed to a cocktail of mild doses of pro-inflammatory cytokines (CK) encountered at disease initiation. To discriminate between inflammation-specific and general responses to cellular stress, hypoxic (1% O_2, HX) and genotoxic (ultraviolet irradiation, UV) stress situations were introduced (**Figure 1A**). Cells grown under hypoxia were assessed for the expression of endogenous and exogenous markers of hypoxia. Added to culture, pimonidazole hydrochloride forms adducts with thiol groups in proteins in cells at low oxygen tension ($pO_2 < 10$ mmHg). Immunofluorescent microscopy analysis revealed the presence of pimonidazole adducts in hypoxic cells (**Figure 1B**). The hypoxia-inducible factor 1 (HIF-1) is a transcriptional regulator of the cellular response to low oxygen levels. Under hypoxic conditions, the subunit HIF-1α associates with the subunit HIF-1β and binds to the hypoxia response element (HRE) of target genes, initiating their expression (42, 43). ELISA analysis showed a 3-fold increase in HIF-1α expression from 9 (7-12) pg/mL [median (range)] for cells grown under normoxic conditions compared to 29 (15-44) pg/mL for cells grown under hypoxic conditions (**Figure 1C**; $p = 0.0143$).

Exposure to experimental stress induced ER stress as revealed by enhanced expression of the phosphorylated form of the subunit alpha of the eukaryotic translation initiation factor 2 (p-eIF-2α) and the transcription factor C/EBP homologous protein (CHOP), two effectors of the unfolded protein response (UPR) to ER stress (**Figure 1D**, **Supplementary Figure 1**). While p-eIF-2α participates to translational attenuation with the aim to restore protein homeostasis in the ER at an early stage of the UPR, CHOP is activated belatedly after prolonged stress and controls cell fate by regulating expression of genes involved in apoptosis. After 30 h of culture, cytokine- and HX-treated cells expressed CHOP in contrast to UV-irradiated cells. This difference might be explained by altered and presumably delayed kinetics of activation of the UPR following DNA damage at random by UV-light that pass through transcriptional and translational steps prior to changes in the proteome.

All treatment conditions engendered apoptosis in MIN6 cells as shown by the significant increases in the percentage of effector caspase-3/7- positive cells (**Figures 1E,F**). The quantitative analysis of fluorescent caspase-substrate cleavage on confocal microscopy images (total > 8,000 nuclei counted for each situation) revealed a low percentage of 1.5 (0-5)% [median and (range)] of caspase-3/7-positive cells in untreated controls. Following exposure to stress, this percentage shifted to 20 (10-34)% for CK-, 5 (3-14)% for UV-, and 5 (1-11)% for HX-treated cells. Live cell imaging was performed to monitor the kinetics of apoptosis in individual cells (**Supplementary Figure 2**). In response to cytokines, caspase-3/7 activity appeared after 5 h of treatment and steadily increased. Close to all cytokine-treated cells became apoptotic by the end of the 30h incubation period in contrast to untreated controls. Collectively, these results demonstrate that stress in our experimental conditions rapidly induces critical executioners of the cellular stress response in MIN6 beta cells cumulating in apoptosis.

To assess downstream effects of cellular stress on the beta cell's secretome, AB, MV and sEV subpopulations were enriched from 30h conditioned MIN6 culture supernatants following a protocol combining differential centrifugation (44), (ultra-) filtration and size-exclusion chromatography (45) steps (outlined in **Figure 2A**). Western blot revealed the presence of vesicular markers i.e. the membrane proteins CD81, CD63, CD9 and flotillin, and the cytosolic protein β-actin in all EV subpopulations (**Figure 2B**, **Supplementary Figure 3**). The ER protein calnexin was present in AB and MV but absent in sEV in line with enrichment in vesicles of endosomal origin in the latter.

Staining with the lipid probe MemBright, recently developed by Collot and colleagues (46, 47), showed a heterogeneous population of round-shaped vesicles in the AB and MV fractions (**Figure 2C**). Cryo-electron microscopy images of MV and sEV clearly ascertained the presence of a lipid bilayer membrane surrounding the vesicles (**Figure 2D**, **Supplementary Figure 4**). AB exceed the upper size limit of cryo-electron tomography (1μm) and have therefore not been analyzed using this technique. TRPS analysis of the EV showed a mode size [median (range)] of 1548 (1368-1790) nm, 510 (455-563) nm, and 76 (61-120) nm for AB, MV, and sEV, respectively. None of the treatments had a significant effect on the mode or mean size of the vesicles (**Figure 2E** and **Table 1**). In healthy beta cells, large EV (AB and MV) represent 93% of the volume and 98% of the protein content of the vesicles all together, but less than 2% of the number of particles (**Figure 2F**). In pro-inflammatory conditions, the secretion of AB, MV and sEV was significantly enhanced as shown by the 5.5-fold, 2.1 and 4.5-fold increases of the number of particles recovered per million of cells, respectively. Commensurate to the rise in the number of vesicles, the volume occupied by the CK-EV (all subtypes) increased 4.0-fold against 2.8-fold for UV and 1.7-fold for HX EV (**Figure 2G**, **Table 1**). In line with the particle size, the number of particles per microgram of protein is much higher in MV (> 25 times) and sEV (>1E4 times) than in AB. As expected, this ratio remained constant in conditions of stress for MV and sEV, but curiously increased in AB derived under conditions of stress (CK vs CTL; $p = 0.0084$). In the absence of noticeable changes in the particle's

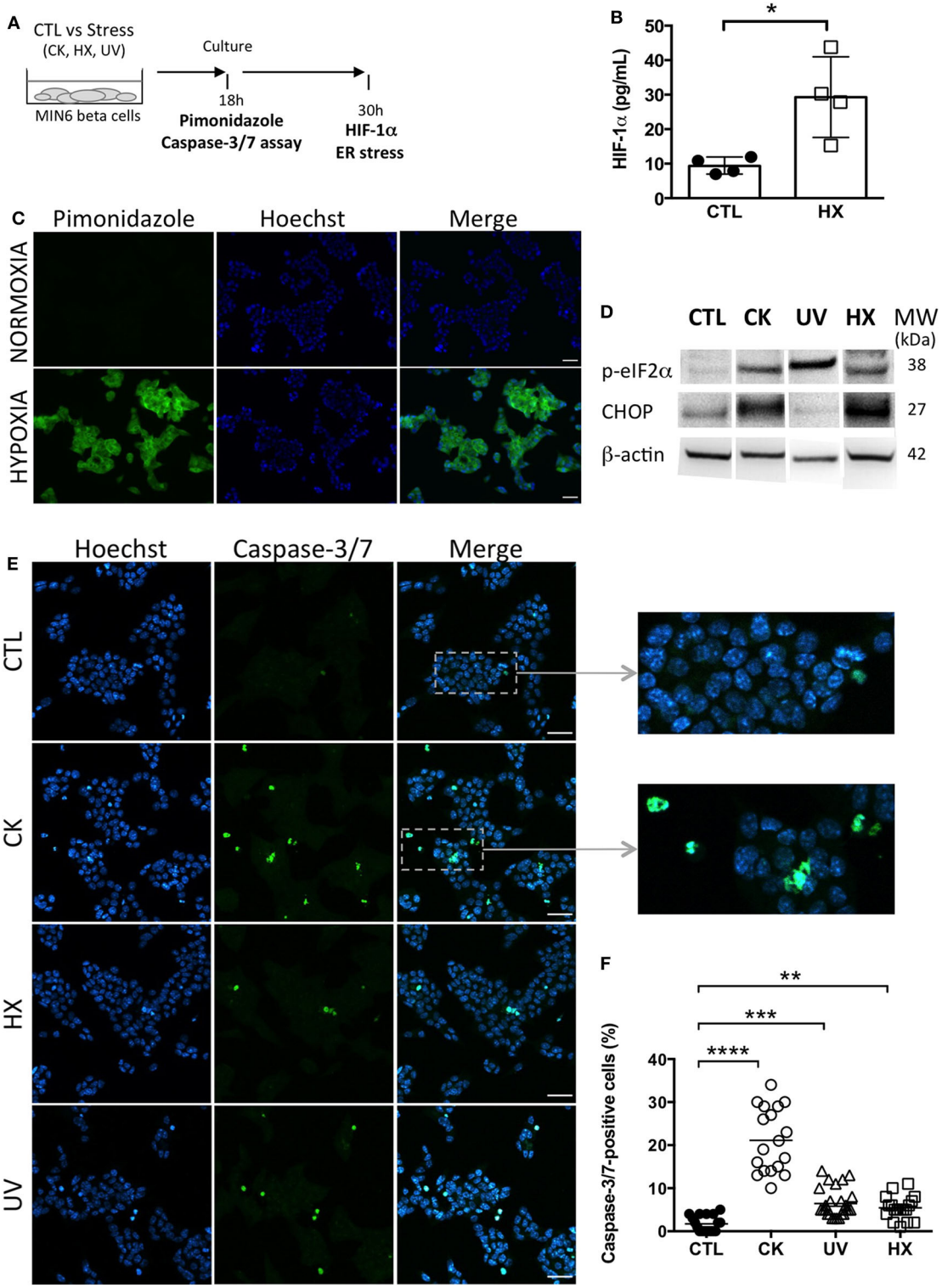


FIGURE 1 | Experimental beta cell stress induces apoptosis. **(A)** After exposure to stress [TNFα, IL-1β, IFN-γ cytokines; CK], hypoxia (HX), ultraviolet (UV)] or not [control (CTL)], MIN6 cells were cultured in 1% FCS exosome-depleted OptiMEM medium. **(B,C)** For cultures grown under normoxic and hypoxic conditions, **(B)** After 24 h of culture, MIN6 cells were treated with 100 μM pimonidazole and incubated for 2 h followed by immunohistochemical detection of pimonidazole adducts (green). Nuclei were counterstained with Hoechst 33342 (blue). Scalebar 30 μm. Representative images of one out of three independent experiments are shown. **(C)** ELISA of the expression of HIF-1α in cells grown under normoxia or hypoxia for 30 h. Data from 4 independent experiments are depicted with median and range.

(Continued)

FIGURE 1 | Mann-Whitney test, one-tailed, $*p < 0.05$. **(D)** After 30 h of culture, expression of markers of ER stress p-eIF2α and CHOP was analyzed by western blotting and then the membranes were reprobed to β-actin. **(E,F)** After 18 h of culture, the cells were stained with CellEvent Caspase-3/7 reagent (green) or Hoechst 33342 (nucleic dye, blue). **(E)** Fluorescence microscopy images of MIN6 cells reveal cells undergoing apoptosis as shown by the presence of caspase-3/7- positive cells. Scale bar 30 μm. **(F)** The quantitative analysis showed a significant increase in the percentage of caspase-positive cells for treated in comparison to untreated control cells. Each data point represents results obtained for one image. Data are compiled from $n = 18$ images (> 8,000 nuclei) per situation from two independent experiments. Kruskal–Wallis test ($**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$).

volumes measured by TRPS, this increase hints to changes in the vesicle's protein content. Cytoplasmic vacuolation and inclusion of organelles and DNA fragments during the process of apoptosis possibly reduced the proportion of proteins in AB from CK-treated in comparison to AB from untreated cells.

Earlier studies provided evidence that human and mouse beta EV contain major auto-antigens of type 1 diabetes such as GAD65, islet-associated antigen 2, Znt8, and insulin (12–15). Out of these, insulin is the most prominent islet autoantigen, highly abundant in beta cells and was used here to ease monitoring of autoantigen partition.

To explore how autoantigens partition into beta EV in normal and pathological conditions, we quantified the amount of total insulin comprising pro-insulin and mature insulin in EV subpopulations by ELISA (**Figure 3A**). Data obtained on EV from healthy cells showed that a majority of vesicle-associated insulin was exported inside large vesicles (**Figure 3B**). The absolute insulin content [median (range)] was in the range of 7 x 10^{-14} (1 x 10^{-14} – 1 x 10^{-12}) g/part. in AB, 1 x 10^{-15} (1 x 10^{-16} – 7 x 10^{-15}) g/part. in MV down to 8 x 10^{-19} (2 x 10^{-19} – 4 x 10^{-18}) g/part. in sEV, in line with the volume of the particles (**Supplementary Figure 5A**). In comparison to AB, a 1.5-fold and 4.6-fold lower median insulin concentration was measured respectively inside MV and sEV, where insulin represented 0.1 (0.0-0.3)% of the vesicles' protein content (**Figure 3C** and **Supplementary Figure 5B**).

Following exposure to stress, insulin export was markedly enhanced in AB and sEV derived from CK-treated cells, whereas no significant changes were perceived in EV derived from cells cultured exposed to hypoxia or UV-irradiation (**Figure 3D** and **Supplementary Figure 5**, **Supplementary Table 1**). This increase in insulin export relied on enhanced EV export, as the concentration of insulin inside the EV subtypes did not change following treatment.

MiRNA may act as adjuvants in immune activation and recently six miRNA with the potential to bind directly to the TLR-7 receptor of innate immunity have been described in MV and sEV (23, 24, 26–28, 48, 49). Here we wanted to investigate how TLR-binding miRNA are sorted into EV and whether their expression in these vesicles changes in situations of stress.

With the aim to compare TLR-binding miRNA expression in an equal amount of cells and large and small EV derived thereof, a synthetic ath-miR-159a was spiked into all samples prior to RNA extraction. After RT-qPCR amplification, relative quantities in samples were normalized with respect to this exogenous control as well as cells or EV derived from untreated control cells (**Figure 4**). The results obtained show a significant up to 3-fold drop in the expression of TLR-binding miRNA in cells in situations of pro-inflammatory stress, in parallel to an up to 13- and 48-fold increase in LEV and sEV, respectively. Although less pronounced, similar trends were observed in cells and LEV obtained under genotoxic and hypoxic conditions. No changes of TLR-binding miRNA expression were observed in sEV under genotoxic and hypoxic conditions. This enhanced TLR-binding miRNA release in vesicles could be explained either by an increase in the release in EV or by a higher concentration of these miRNA inside the vesicles. To answer this question, the RT-qPCR data was further normalized to the number of particles present in the sample as determined by TRPS. The results presented in **Supplementary Figure 6**, revealed an evened out expression of TLR-binding miRNA in LEV in all situations. In contrast, 4–5-fold higher quantities of all TLR-binding miRNA except for miR-29b were detected in sEV secreted under proinflammatory conditions. Taken together, these data suggest that beta cell stress and in particular, the exposure of beta cells to cytokines, favors export of immune stimulatory miRNA into EV and enrichment in sEV.

Cytokines are well-known soluble mediators in cell-to-cell communication, however evidence exist that an important fraction of biologically active cytokines are released from tissues in association with EV either bound to the surface or encapsulated in the lumen of the vesicles (50). Owing to cytokine treatments performed in our EV production workflow, we performed a mouse 13-plex CBA to assess for interferon (IFNβ, IFNγ, interleukin (IL-1α, IL-1β, IL6, IL-10, IL-12p70, IL-17A, IL-23, IL-27), granulocytes macrophage colony stimulating factor (GM-CSF), TNFα and monocyte chemoattractant protein-1 [MCP-1; also called chemokine (C-C motif) ligand 2 (CCL2)] cytokine expression in the different subpopulations of beta EV. Prior to analysis, the EV were incubated in 0.5% Triton X-100 for 30 minutes and sonicated for 1 min. to assess for internal as well as surface-associated cytokines. Six cytokines were detected in subpopulations of MIN6 beta EV (**Figure 5** and **Supplementary Table 2**). Seven cytokines were below detection levels in all vesicles. None of the EV secreted by untreated controls exhibited the exogenous cytokines TNFα, IFNγ or IL-1β (**Supplementary Table 2**). Trace amounts of MCP-1 and IL-23 were detected in CTL-AB and CTL-MV. Following exposure to picomolar concentrations of cytokines in the initial culture medium, MIN6 release femtograms of IFNγ, TNFα and IL-1β per million of cells in AB and, to a lesser extent, in MV. IFNγ was not detected in sEV and quantities close to detection thresholds of IL-1β and TNFα were detected in only 1 out of four and 3 out of four samples, respectively. Interestingly, MIN6 cytokine treatment stimulated a dramatic increase in the expression [median (range)] of MCP-1 in AB [358 (281– 415.5) fg/E6], MV [127.5 (65.1 – 208.8) fg/E6 cells] and sEV [16.4 (3.6 – 25.4) fg/E6 cells] and of IL-27 in AB [24.1 (15.7 – 33.4)

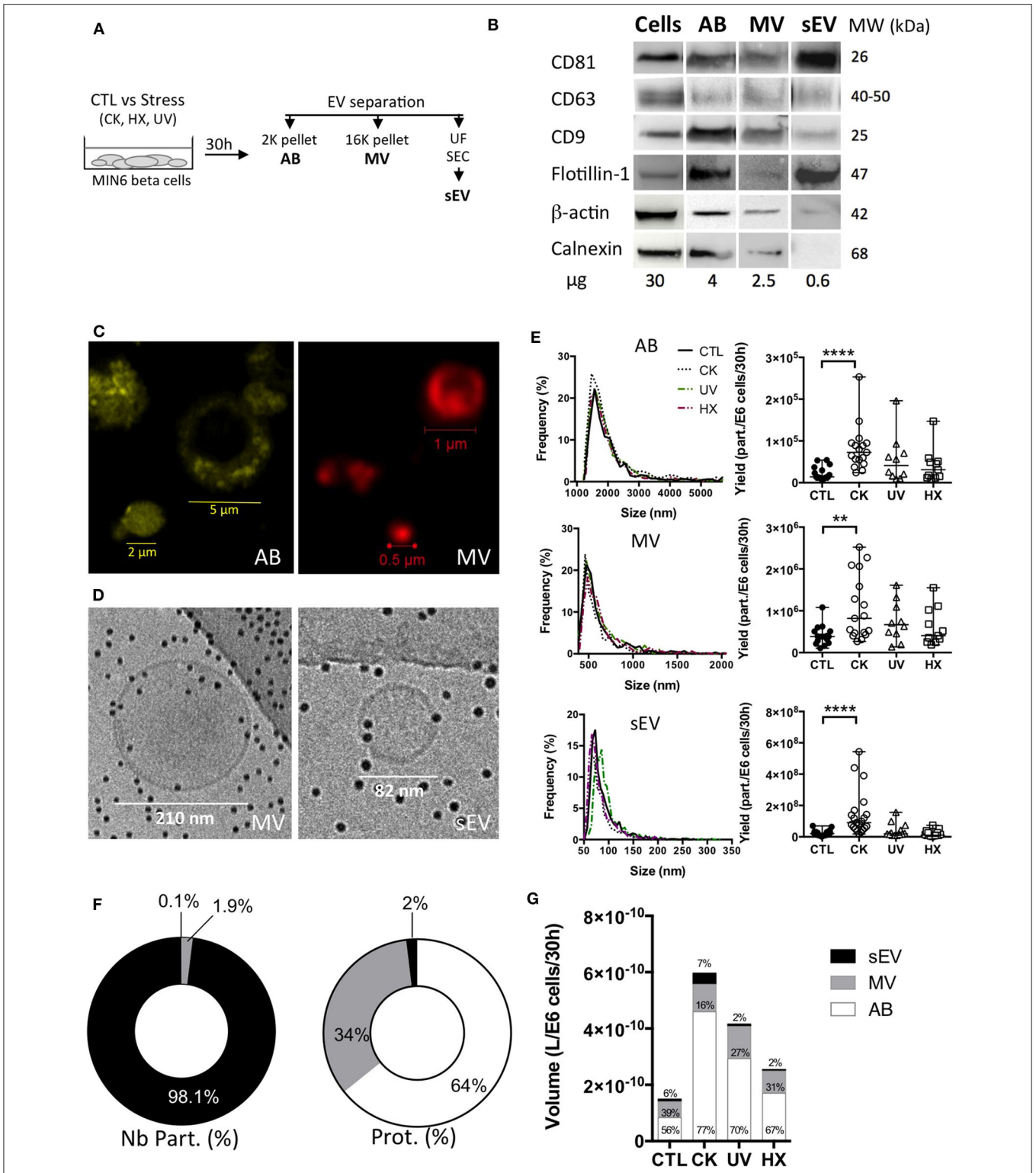


FIGURE 2 | Apoptotic beta cells release a heterogeneous population of EV. **(A)** After exposure to stress, MIN6 cells were cultured for 30 h prior to EV separation by differential centrifugation, ultrafiltration (UF) and size-exclusion chromatography (SEC). **(B)** Representative Western blot images of markers of extracellular vesicles (membrane CD81, CD63, CD9, Flotillin-1, cytosolic β-actin) or cells (Calnexin). **(C)** Confocal images of EV from untreated MIN6 cells stained with MB-Cy3 (AB) or MB-Cy5 (MV) **(D)** Cryo-electron microscopy images of MV and sEV. **(E–G)** The particle size distribution of EV subpopulations was determined by TRPS analysis.

(Continued)

FIGURE 2 | Histograms show the particle size distribution of samples from one representative experiment. Scatter dot plots represent the number of particles recovered per million of cells (data from $n = 10–21$ independent experiments; median with range). Yields of EV obtained from MIN6 cells after treatment were compared to the yield from untreated controls using the Kruskal-Wallis test (**$P < 0.01$ ****$P < 0.0001$). **(F,G)** Relative quantities of material released inside EV based on TRPS and Bradford protein content analyses of extracellular vesicles harvested per million of MIN6 cells after 30 h of culture. Relative volumes occupied by vesicle subtypes are estimated based on mean sizes and concentrations measured by TRPS. **(F)** Percentage of numbers of particles (Nb part.) and total protein of EV subtypes derived from untreated control cells. Results are depicted as median percentages from $n = 7–9$ replicates from independent experiments. **(G)** Volumes (median) measured for EV from treated and control MIN6 cells. Results from $n = 10–17$ independent experiments are shown.

TABLE 1 | Physical properties of beta EV.

		Mode (nm)	Mean (nm)	Yield (Nb part./E6 cells)	Fold increase volume	Nb part./μg protein
AB	CTL	1,548 (1,368–1,790)	2,108 (1,826–2,370)	1.4E4 (0.6E4–5.4E4)	1.0	4.4E4 (1.3E4–15.0E4)
	CK	1,606 (1,448–1,904)	2,162 (2,053–2,687)	7.6E4 (2.4E4–25.3E4)****	5.5	10.6E4 (4.2E4–85.6E4)**
	UV	1,618 (1,500–1,899)	2,153 (2,065–2,356)	5.6E4 (0.9E4–19.6E4)	3.5	8.1E4 (2.0E4–36.1E4)
	HX	1,580 (1,463–1,799)	2,145 (2,084–2,247)	2.8E4 (0.9E4–14.7E4)	2.0	8.4E4 (2.4E4–27.0E4)
MV	CTL	510 (455–563)	676 (637–787)	3.8E5 (1.1E5–10.8E5)	1.0	1.1E6 (0.6E6–4.9E6)
	CK	498 (458–573)	623 (587–745)	8.2E5 (2.7E5–25.2E5)**	1.7	2.0E6 (1.0E6–11.3E6)
	UV	512 (472–578)	720 (660–841)	6.7E5 (1.4E5–16.1E5)	2.0	1.3E6 (0.3E6–5.7E6)
	HX	536 (486–572)	717 (605–798)	4.1E5 (1.9E5–15.5E5)	1.4	1.9E6 (0.4E6–4.9E6)
sEV	CTL	76 (61–120)	101 (78–140)	2.0E7 (2.0E6–6.9E7)	1.0	1.6E9 (0.1E9–4.1E9)
	CK	75 (61–124)	92 (72–140)	9.1E7 (1.8E7–54.3E7)****	4.3	2.0E9 (0.2E9–13.4E9)
	UV	84 (62–118)	100 (81–141)	1.9E7 (8.8E6–15.6E7)	1.0	1.5E9 (0.07E9–24.6E9)
	HX	82 (67–118)	101 (85–143)	1.0E7 (2.8E6–7.4E7)	0.7	1.0E9 (0.02E9–11.6E9)

All values are listed as median (range) from n = 9–21 independent experiments. Nb part: number of particles. Kruskal-Wallis test compared to EV from untreated controls. **$P < 0.01$, ****$P < 0.0001$.

fg/E6 cells]. Cytokine profiles of EV derived from MIN6 cells exposed to hypoxia or UV-irradiation were similar to profiles from untreated cells (data not shown).

APC such as dendritic cells and macrophages have been identified as recipient cells for beta EV uptake *in vitro* and *in vivo* (12, 13, 51, 52). For side-by-side comparisons of the potential of EV subtypes to modulate APC function, primary NOD bmDC were exposed to MIN6-derived beta EV for 18 h followed by flow cytometry analysis of the expression of MHC-II and co-stimulatory CD86 and CD40 molecules (**Figure 6**). AB derived from cytokine-treated beta cells induced a modest albeit significant up-regulation of CD40 and MHC II expression. CD86 expression remained unchanged despite a wider variation of expression. None of the MV and sEV modulated the expression of co-stimulatory molecules in bmDC (**Figure 6A** and data not shown).

The concentration of cytokines in supernatants of murine bmDC and RAW264.7 macrophages exposed to EV in culture was assessed by CBA and ELISA (**Figure 7**). In bmDC, no significant influence of the EV treatments was observed on the concentrations of IL-1β, IL-1β, IL-6, IL-10, IL-12p70, or IL-23 whose levels remained low in culture supernatants, close to detection thresholds (**Figure 7A** and data not shown). Among cytokines expressed in beta EV derived under inflammatory situations, MCP-1 was detected in bmDC cultures with AB and MV derived from MIN6 cells cultured under inflammatory conditions (**Figure 7B**). MCP-1 concentrations measured in culture supernatants are 2–4 times lower than concentrations calculated for AB input into these culture, supporting the idea of an essentially passive carry-over of MCP-1. In contrast, IL-27, which was highly expressed in CK-AB, was below detection thresholds in bmDC culture supernatants (data not shown) suggesting differential kinetics of MCP-1 and IL-27 uptake, recycling or activity in bmDC. Alternatively, sustained expression of MCP-1 in culture supernatants could be explained by *de novo* cytokine production by bmDC. All AB (except UV-AB) and sEV derived under inflammatory conditions led to increased levels of TNFα in bmDC culture supernatants, superior to TNFα amounts provided by these vesicles. Though CK-MV expressed 2.5-fold higher levels of TNFα than CK-sEV, no differences in the concentration of TNFα was observed in bmDC supernatants in the presence of CK-MV in comparison to CTL-MV (**Figure 7C**). Co-incubation of murine RAW264.7 macrophages with AB, MV as well as sEV led to increased TNFα supernatant concentrations for EV obtained from cytokine-treated MIN6 cells, the amounts of which cannot solely be explained by passive carry-over of EV-associated TNFα. For EV from beta cells in hypoxia, AB were also able to significantly enhance TNFα secretion (**Figure 7D**; $p = 0.0122$). For all EV from UV- and HX-stressed beta cells, a tendency of TNFα induction was visible. As inflammatory stress was the most potent inducer of EV release in our hands (**Figure 2**), the more pronounced immune effects might be caused by a higher ratio of EV to target immune cells or a minimal concentration of EV necessary for immune activation. Taken together, our results reveal modest direct or indirect activation of dendritic cells and macrophages by beta EV.

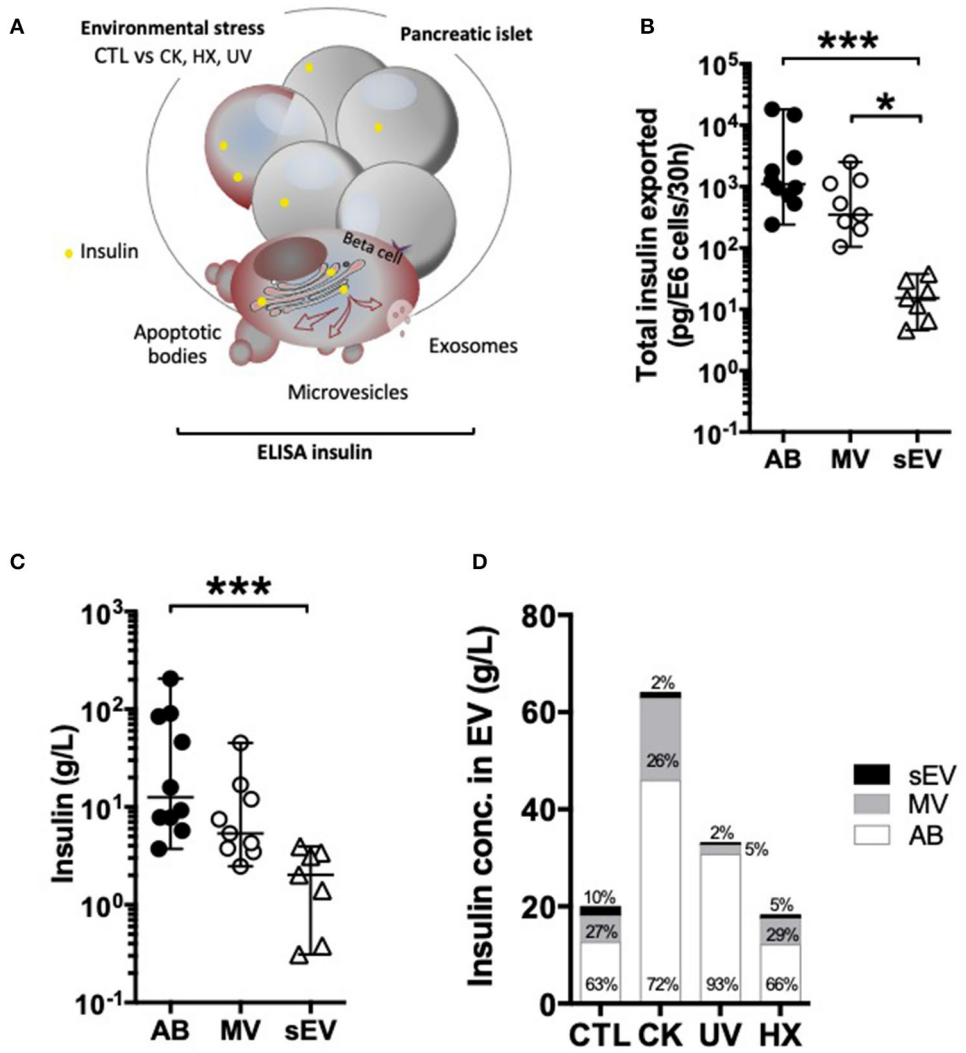


FIGURE 3 | Differential partition of the autoantigen insulin inside EV. **(A–D)** After 30 h of culture, EV were collected from supernatants from CTL, CK, UV, or HX MIN6 cells and assessed for total insulin (pro-insulin and mature insulin) content by ELISA. **(B,C)** On EV derived from untreated control cells (CTL) **(B)** Quantities of insulin measured inside AB, MV and sEV collected per million of CTL cells. **(C)** Insulin concentrations measured in association with AB, MV, and sEV from CTL MIN6 cells. **(D)** Evolution of sorting of insulin toward EV following exposure to stress. Data from $n = 7–11$ independent experiments are shown with median and range and compared using the Kruskal-Wallis test (*$P < 0.05$ ***$P < 0.001$).

DISCUSSION

Living cells release heterogeneous populations of EV that constitute a means for surrounding and distant tissue crosstalk. Beta sEV have been shown to drive innate and adaptive prodiabetogenic immune responses, but the functional diversity of the beta secretome as a whole, and the impact of beta cell stress on the beta EV repertoire have not been explored yet.

We observe that experimental exposure of MIN6 beta cells to either inflammatory cytokines, low oxygen tension or UV-irradiation rapidly induces ER-stress and subsequently apoptosis. While p-eIF-2a, indicative of translational attenuation is observed in all situations of stress, the apoptosis-mediating transcription factor CHOP is barely detected in UV-treated MIN6 cells. This is in line with earlier observations that DNA damage through UV irradiation alone is insufficient to induce CHOP expression (53). Although low doses of cytokines were used here in comparison to similar beta cell studies (17, 54, 55), the percentage of caspase-3/7 positive beta cells were 4-fold higher in CK-treated cells than in cells following UV irradiation or cultured under hypoxia, illustrating the particular propensity of beta-cells to undergo apoptosis in response to inflammatory stressors (56).

Stress and inflammation have been repeatedly reported to enhance EV secretion, including from beta cells (12, 57). Here, quantitative side-by-side comparisons of EV subtypes isolated from an equal amount of beta cells, reveal a consistently higher release of EV exclusively under inflammatory conditions. To

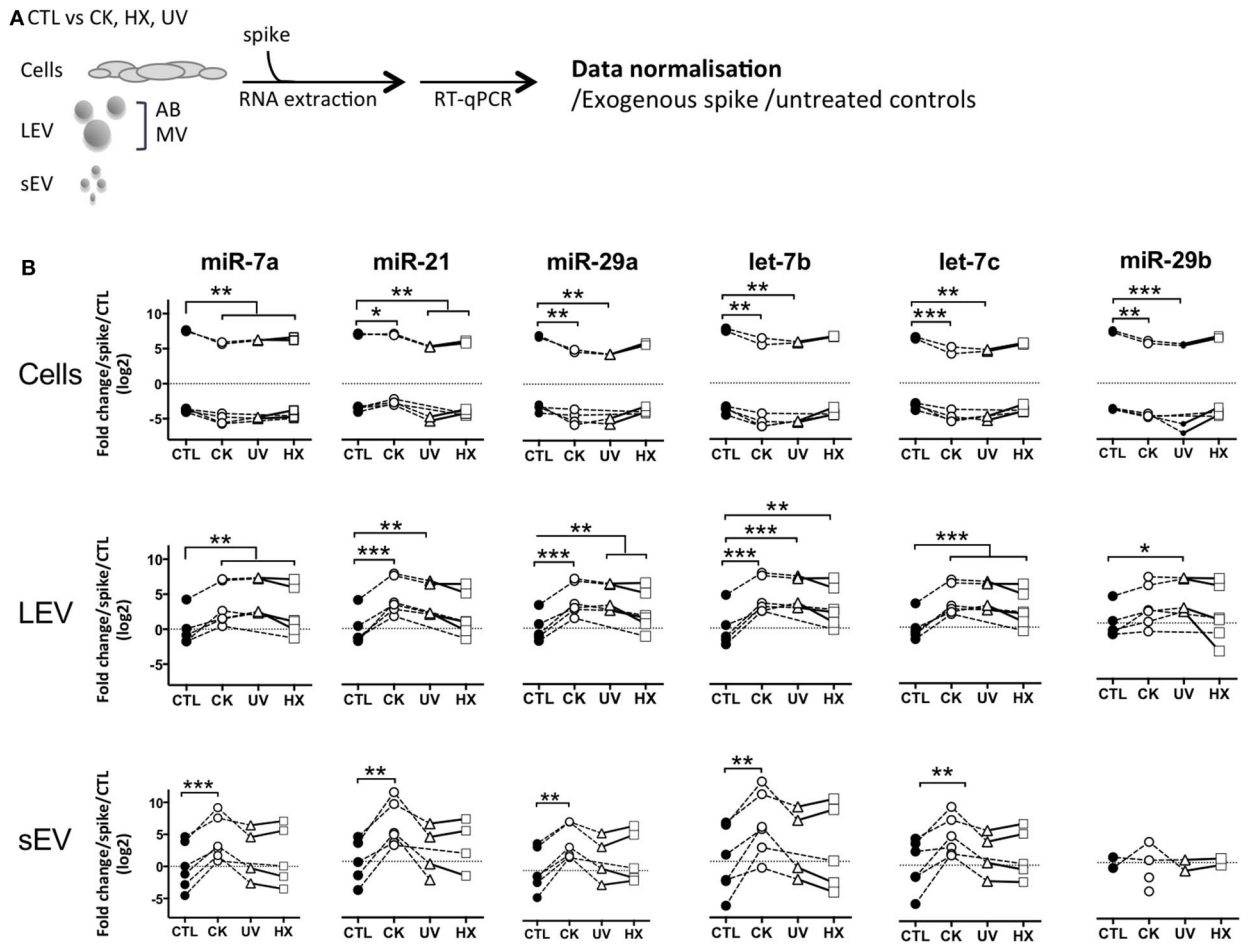


FIGURE 4 | Beta cell stress favors export of TLR-binding miRNA in EV. **(A)** Following exposure to stress, MIN6 cells were cultured for 30 h followed by isolation of large EV (comprising AB and MV) and sEV. All samples were spiked with an exogenous control prior to total RNA extraction and processed for quantitative RT-PCR. After amplification, relative quantities were normalized with respect to the spike and untreated controls. **(B)** Quantitative RT-PCR analysis of miRNA expression in a fixed number of cells and EV derived thereof. Individual replicates from 4 to 6 independent EV isolations are represented as fold-changes compared to mean expression values measured in untreated controls. Tukey's range test $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

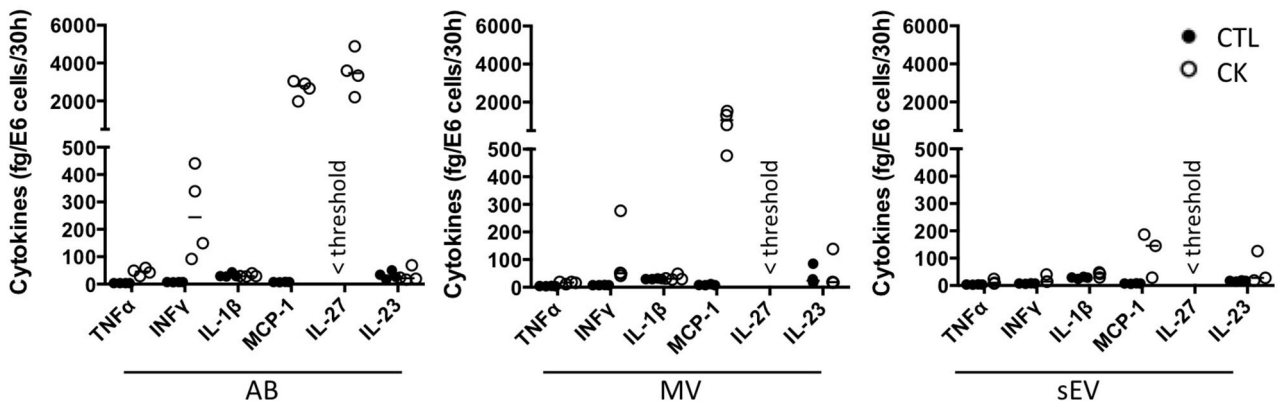


FIGURE 5 | Cytokine profile of beta EV. Multiplex CBA monitoring of cytokine expression in association with EV collected from 30 h culture supernatants from cytokine-treated MIN6 cells and untreated controls. Data (with median) from 3 to 4 independent experiments are shown.

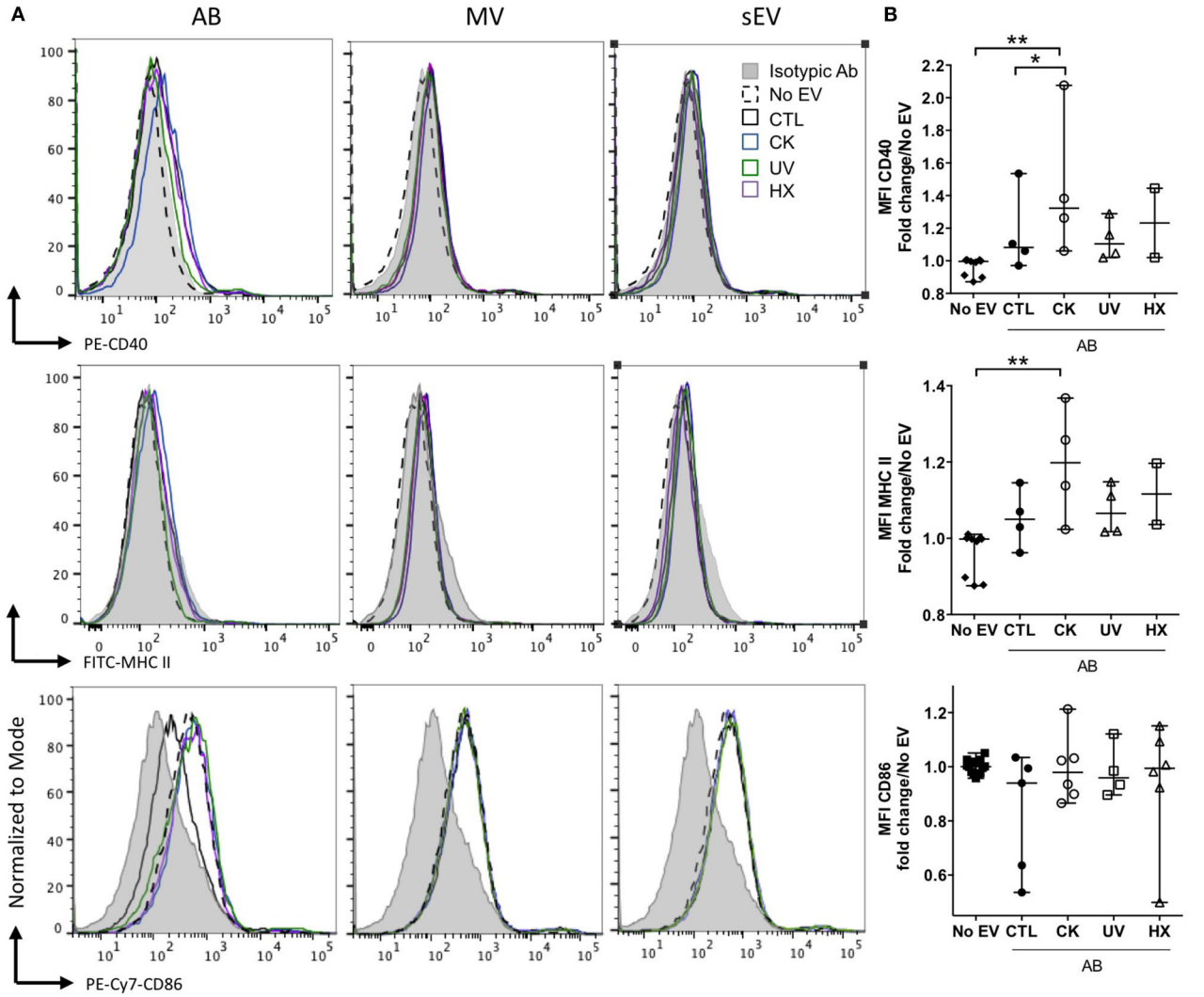


FIGURE 6 | Modulation of co-stimulatory molecules in NOD bmDC by beta AB. NOD_{Shi} mice derived bmDC were incubated for 18 h with AB, MV or sEV derived from 8×10^7 MIN6 cells. **(A)** Flow cytometric analysis of the expression of the CD40, MHC II, and CD86 activation markers. Histograms with dashed lines represent bmDC treated with AB derived from cytokine beta cells. Histograms with solid lines represent bmDC treated with AB from untreated beta cells. Gray histograms represent isotypic controls. Data from one representative out of 2–5 independent experiments are shown. **(B)** Scatter plots show the median fluorescence intensity (MFI) of co-stimulatory molecules expression in bmDC after AB treatment normalized to No EV controls (median with range). Tukey's range test $^*P < 0.05$ and $^{**}P < 0.01$.

what extent, the 5-, 2-, and 4-fold increases in the number of particles observed for AB, MV, and sEV, respectively, impact downstream immune responses presumably depends on the nature of their cargo.

Indeed, in response to stress, cancer cells secrete EV which have been shown to contribute to survival of surrounding cancer cells and drug resistance (58). In contrast, sEV from cytokine-treated beta cells induce beta cell apoptosis in naïve beta cells (17) suggesting an EV-mediated spread of inflammatory cellular constituents. Following cytokine exposure, it has been shown that chaperones of the UPR promoting DAMP-signaling, namely calreticulin, Gp96, ORP150 and the heat-shock protein HSP-90α are packed into beta sEV (12, 54). Beta EV have also attracted interest for their aptitude to transport self-antigens (12–15). In the present study, the partition of the highly abundant insulin protein was monitored in EV subpopulations derived under normal and pathological conditions. In our hands, 1.5% of untreated MIN6 cells continuously undergo apoptosis in culture. These apoptotic cells release AB containing 91% of the particulate secretome's insulin content in line with the role of AB in the disposal of cellular material in efferocytosis (59–62). Exposure to inflammatory triggers up-regulates not solely the number of vesicles released, but also the absolute amount of insulin exported with significantly higher levels of insulin measured in association with EV produced by cytokine-treated cells. Our results converge with others obtained for TLR-binding miRNA expression inside large and small EV. Inflammatory cytokines and to a lesser extent hypoxia and UV-irradiation

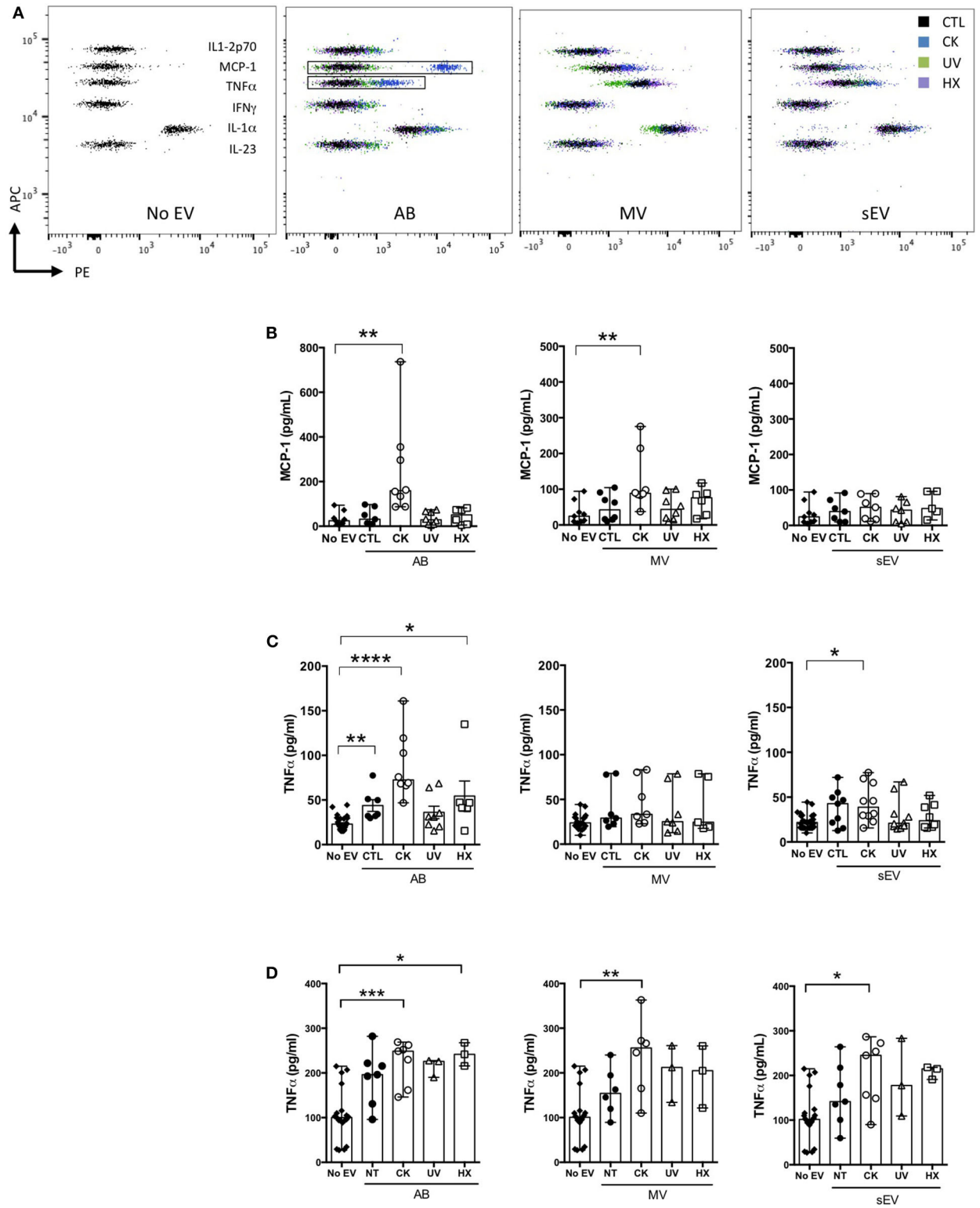


FIGURE 7 | Modulation of cytokine secretion profiles in innate immune cells by beta EV. **(A–C)** NOD_{Shi} mice derived bmDC were incubated for 18 h with AB, MV, or sEV derived from 8×10^7 MIN6 cells **(A,B)** CBA of cytokine concentration in bmDC culture supernatants. **(A)** Dot plots of fluorescence intensity of beads from one representative experiment are shown. **(B)** CBA of MCP-1 in culture supernatants from bmDC treated with AB or No EV controls. Data from 6 to 7 independent experiments are shown (median with range). **(C)** TNFα supernatant levels as confirmed by ELISA. Data from 6 to 8 independent experiments are shown. **(D)** RAW264.7 macrophages were treated for 18 h with AB, MV or sEV derived from 8×10^7 MIN6 cells. TNFα cytokine levels in culture supernatants were measured by ELISA. Data from 3 to 6 independent experiments are shown. Kruskal Wallis test $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

promote TLR-binding miRNA efflux from the cell. Interestingly, this increase relies on the enhanced secretion of LEV with an unchanged miRNA content in contrast to enhanced secretion paralleled by a rise in the relative quantities of these miRNA sequences packed inside individual sEV. Taken together, our data provide strong evidence that sorting of immunogenic material inside subpopulations of EV is not a random process and is profoundly altered in inflammatory settings.

MIN6 beta EV in the steady state contain low levels of MCP-1 and IL-23 and undetectable levels of a panel of eleven other cytokines involved in pathways of inflammation. Experimental exposure of MIN6 cells to inflammatory cytokines engenders drastic changes in the expression of the same cytokines in large AB and MV, but also of *de novo* produced MCP-1 (in AB, MV, and to a lower extent in sEV) and IL-27 (AB) cytokines. Passive exogenous cytokine carry-over is obviously a concern in the interpretation of immune functions of EV. However, it has to be stressed that beta cells facing immune insults *in situ*, most likely discard cytokines of beta or immune cell origin in an analogous manner that is to say inside large rather than small vesicles according to our data.

Several studies, including genome-wide association studies (63–65), demonstrate pathogenic roles of the cytokine IL-27 in T1D. Transgenic NOD IL-27 receptor knockout mice are resistant to disease and blockade of IL-27 delays T1D onset in NOD mice (66, 67). MCP-1 is a chemokine involved in immune cell recruitment. Exported in exosomes, MCP-1 has been shown to contribute to inflammation in nephropathies (68, 69). In the context of T1D, chemotaxis assays showed that subnanomolar amounts of MCP-1 produced by beta cells are sufficient to attract monocytes (70). It has been shown earlier, that mouse and human islet cells constitutively express MCP-1 and produce high levels of MCP-1 peaking at 6 h of incubation in response to proinflammatory cytokines (71, 72). In islet transplantation, MCP-1 is inversely correlated to islet graft function (70, 73) and attempts to block MCP-1 signaling successfully improve graft survival (74). Furthermore, T-lymphocyte exosomes induce MCP-1 expression and apoptosis in beta cells (75) illustrating the importance of MCP-1 in beta cell inflammation and failure. MCP-1 stimulation on its own results in aberrant sorting of immune regulatory miRNA into extracellular vesicles (76) in line with observations made on TLR-binding miRNA in our study. It is thus conceivable that molecular mediators of inflammation as chemokines and immunostimulatory miRNA establish and mutually maintain inflammation.

Added to culture of bmDC derived from diabetes-prone NOD mice, EV from cytokine-treated beta cells up-regulate moderately the surface expression of MHC class II and co-stimulatory CD40 molecules. In these experiments, an EV donor to recipient ratio of 1:200 was used, which would be equivalent to 5 DC in an averaged sized islet containing 1,000 beta cells, a plausible proportion in the inflamed pancreas at disease initiation. EV from CK-treated MIN6 cells exert the strongest immune effects, which could be due to cumulative effects of cargo quantity (autoantigens, proinflammatory miRNA, endogenous cytokines), increased EV release and cytokine carry-over. At least two facts argue against cytokine carry-over as the only responsible for the observed immune effects. First, AB derived under hypoxia devoid of EV-associated cytokines also significantly induced TNFα secretion in RAW264.7 macrophages. Second, IL-27 highly expressed in CK-AB was not detected in bmDC culture supernatants. Lastly, we showed earlier that TNFα secretion in RAW264.7 macrophages induced by sEV derived from untreated MIN6 cells is positively correlated to the amount of particles in culture (26, 51).

The relevance of these quantitative and qualitative differences of subsets of apoptotic beta EV have to be weighted with regard to the interplay of these vesicles with cellular effectors of immunity *in vivo*. Obviously, enhanced EV release in situations of stress engenders higher EV to immune cell ratios. This fact should be considered in EV biological activity assays, which are frequently based on treatments with a constant number of particles. AB are known to express "find me" and "eat me" signals leading to rapid elimination by patrolling phagocytes (77). Conceivably, AB from stressed beta cells constitute a critical source of chemo-attractants, beta self-antigens and danger signals that could infer with the otherwise immune silent elimination of the dead by efferocytosis. In contrast, nanosized vesicles such as sEV and small MV have half-lives of minutes to hours *in vivo* (78). They are in the ideal size range for transport in interstitial fluids and have been shown to efficiently diffuse to secondary lymphoid organs as spleen and draining lymph nodes (79–81). Thereby, MV and sEV from inflamed pancreatic islets could have implications in immune regulation by aberrant autoantigen and immune-stimulatory miRNA expression at nearby as well as distant sites. Taken together, our findings highlight the profound impact of inflammation in comparison to other stressors on the beta EV repertoire. Centered on stress, the induction of markers of activation and mediators of inflammation (with the exception of IL-10) by beta EV are analyzed in the present work. Further investigations on primary mouse and human islet and immune cell cultures *in vitro* and in pre-diabetic NOD mice *in vivo* are required to dissect the mechanisms of potential protective vs. pathological roles of EV subspecies from healthy and stressed beta cells in T1D development.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Pays de la Loire regional committee on ethics of animal experiments (APAFIS#9871).

AUTHOR CONTRIBUTIONS

KG, LB, DJ, J-MB, GM, and SB designed the experiments. KG, LB, ML, and SB produced and characterized the EV and performed functional assays. DJ carried out immunohistochemical analyses. RF and LD did the confocal microscopy analyses. AD carried out cryo-tomography analyses. All authors contributed to data

analysis. GM performed statistical analysis with RStudio. KG, SB, GM, and J-MB wrote the paper. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01814/full#supplementary-material

Supplementary Figure 1 | Original Western blot images of ER stress markers in MIN6 beta cells. After 30 h of culture, 40 μg of cellular protein lysates were blotted and the expression of markers of ER stress **(A)** p-eIF2α and **(B)** CHOP was analyzed by western blotting before **(C)** reprobing of the membranes to β-actin.

Supplementary Figure 2 | Time-lapse of caspase-3/7 activation. For monitoring of kinetics of apoptosis in live cells, a 30 h time-lapse acquisition was performed on CK and untreated control MIN6 cells on a LSM780 confocal microscope (Zeiss, Oberkochen, Germany) in a 5% CO_2, 20% O_2, 37°C atmosphere. Apoptosis was assayed by fluorescent caspase-3/7 substrate cleavage staining. Briefly, 3×10^5 cells/cm^2 MIN6 cells were cultured in eight-chamber labteks with coverslips. After overnight culture, cells were switched to OptiMEM 1% FCS production medium and exposed to cytokines or left untreated and 2 mM caspase-3/7 detection reagent (Fisher Scientific) were added. Images were captured every 15 min for 30 h.

Supplementary Figure 3 | Original Western blot images of cellular and EV markers. After 30 h of culture, EV were harvested from culture supernatants and processed for protein analysis. For all treatment conditions, equal volumes of protein lysates were blotted e.g., 30, 30, and 50 μL for AB, MV, and sEV, respectively. For cells, a same amount of proteins was loaded for all situations. Western blotting was performed to analyse the expression of **(A)** CD81, **(B)** CD63, **(C)** CD9, (**D)** Flotillin-1, **(E)** β-actin and **(F)** Calnexin.

Supplementary Figure 4 | Cryo-electron microscopy of EV from untreated MIN6 cells. Images of entire vesicle of MV and sEV are represented in the top row with zooms on inserts depicted in the bottom row. Images were acquired at a nominal magnification of x 29,000.

Supplementary Figure 5 | Differential partition of the autoantigen insulin inside EV. After 30 h of culture, EV were collected from supernatants from MIN6 cells with **(A,B)** no treatment **(C,D)** all treatment situations and assessed for total insulin (pro-insulin and mature insulin) content by ELISA. **(A)** Absolute quantities of insulin measured inside AB, MV and sEV reported to the number of particles. **(B)** Percentage of insulin out of the total protein content in AB, MV, and sEV. **(C)** Sum of quantities of insulin released inside AB, MV and sEV per million of producer cells. **(D)** Concentration of insulin inside EV. Data from $n = 7$–11 independent experiments are shown with median and range and compared using the Kruskal–Wallis test ($^*P < 0.05$ $^{**}P < 0.01$ $^{****}P < 0.0001$).

Supplementary Figure 6 | Beta cell stress favors export of TLR-binding miRNA in EV. **(A)** Following exposure to stress, MIN6 cells were cultured for 30 h followed by isolation of large EV (comprising AB and MV) and sEV. All samples were spiked with an exogenous control prior to total RNA extraction and processed for quantitative RT-PCR. After amplification, relative quantities were normalized with respect to the spike, the number of particles as determined by TRPS analysis and untreated controls. **(B)** Quantitative RT-PCR analysis of miRNA expression in a fixed number of cells and EV derived thereof. Individual replicates from 4 to 6 independent EV isolations are represented as fold-changes compared to untreated controls. Tukey's range test $^*P < 0.05$, $^{**}P < 0.001$ and $^{***}P < 0.001$.

Supplementary Table 1 | EV- associated insulin.

Supplementary Table 2 | EV- associated cytokines.

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Extracellular miR-574-5p Induces Osteoclast Differentiation via TLR 7/8 in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation and joint destruction. Cell-derived small extracellular vesicles (sEV) mediate cell-to-cell communication in the synovial microenvironment by carrying microRNAs (miRs), a class of small non-coding RNAs. Herein, we report that sEV from synovial fluid promote osteoclast differentiation which is attributed to high levels of extracellular miR-574-5p. Moreover, we demonstrate for the first time that enhanced osteoclast maturation is mediated by Toll-like receptor (TLR) 7/8 signaling which is activated by miR-574-5p binding. This is a novel mechanism by which sEV and miRs contribute to RA pathogenesis and indicate that pharmacological inhibition of extracellular miR-574-5p might offer new therapeutic strategies to protect osteoclast-mediated bone destruction in RA.

Keywords: TLR7/8, miR-574-5p, extracellular vesicle (EV), osteoclast differentiation, rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common systemic autoimmune diseases, characterized by synovial inflammation and the destruction of cartilage and bone (1). The pathogenesis of RA is a consequence of a complex interplay between genetic and environmental risk factors, which lead to the loss of immunological tolerance and the activation of innate and adaptive immune responses. Increased osteoclast (OC) differentiation and activation distrupts bone homeostasis in the course of RA (2) by altering the delicate coupling mechanisms between bone formation and resorption (3, 4).

The synovial fluid of affected joints contain high amounts of extracellular vesicles (EV) (5). These are membrane vesicles of endocytic origin that are actively secreted by almost all cell types into biofluids (6) and may play an important role in the pathogenesis of RA (7). A subpopulation of EV, termed small extracellular vesicles (sEV, 50–200 nm in diameter) (8), are involved in cell-to-cell communication in the microenvironment of arthritic joints (9). Among other biologically active substances, sEV contain microRNAs (miRs), a class of small non-coding RNAs (10). MiRs bind in a sequence specific manner to their target messenger RNA (mRNA) and repress gene expression (11, 12). In addition to this, miRs can also activate gene expression by acting as decoys to RNA-binding proteins, thus antagonizing their functions (13, 14). Recently, an alternative function of extracellular miRs has been described, based on their ability to induce innate immune responses.

It was shown that sEV delivered miR-29b and miR-21 can bind and activate toll-like receptor 7/8 (TLR7/8) in human lung cancer (15). However, the detailed mechanism of TLR7/8 activation by miRs is not fully understood and the pathological consequences of this pathway have not been analyzed in RA.

In this study we demonstrate that sEV isolated from the synovial fluid of RA patients significantly enhanced the differentiation of OCs *in vitro*. Moreover, we were able to attribute this effect to sEV delivered miR-574-5p, which significantly increased osteoclastogenesis by activating TLR7/8 signaling. Overall, this study indentified extracellular miR-574-5p as a critical mediator in the pathogeneis of RA and indictes it as a promising new target for RNA therapeutics against bone destruction.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions

The human embryonal kidney cell line (HEK 293) (DMSZ, ACC 035) and HeLa (DMSZ, ACC57) cells were cultured in Dulbecco's modified Eagle medium (DMEM, Life Technologies, ThermoFisher Scientific, Waltham, USA) with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Life Technologies, ThermoFisher Scientific, Waltham, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate (PAA the Cell Culture Company, Cölbe, GER). Synovial fibroblasts (SFs) of RA patients were cultured as described previously (16). This study was approved by the Institutional Ethical Committee (Solna, Stockholm, Sweden ethical number 2009/1262-31/3) and is in compliance with all ethical standards and patients' consent according to the Declaration of Helsinki. Cell culture was carried out in a humidified atmosphere of 5% CO_2 at 37°C. SFs were seeded at a density of 5×10^4 cells/well in 24-well plates and cultured in DMEM-medium supplemented with EV-free FBS (Gibco, Life Technologies, ThermoFisher Scientific, Waltham, USA), 100 U/ml penicillin 100 µg/ml streptomycin and 1 mM sodium pyruvate for 24 h before they were stimulated with 10 ng/ml ml interleukin (IL)-1β (Sigma-Aldrich, Darmstadt, GER) or tumor necrosis factor α (TNF α) (Immuno Tools, Friesoythe, GER) for further 24 h.

sEV Purification

One milliliter of cell culture supernatant, 500 µl of 1:2 diluted serum (with phosphate buffered saline, PBS, Sigma-Aldrich, Darmstadt, GER) or synovial fluid from RA patients were centrifuged at 2,000 xg at room temperature for 20 min. The synovial fluid was pre-treated with hyaluronidase (1,500 U/ml; Sigma-Aldrich, Darmstadt, GER) for 15 min at 37°C, before sEV were isolated. The supernatant was centrifuged at 21,000 xg in 1.5 ml polypropylene tubes (Beckman Coulter, Brea, USA) at 4°C for 60 min in a L7-65 ultracentrifuge using rotor 70.1.Ti (Beckman Coulter, Brea, USA) to remove large membrane vesicles. The supernatant was transferred in a new polypropylene tube and centrifuged at 100,000 xg at 4°C for 60 min. The supernatant was discarded. The remaining pellet was resuspended in PBS. Quantity of purified sEV was determined on protein level via UV-Vis spectroscopy to ensure treatment with equal amounts of sEV between sEV-treatment experiments. SEV were used directly or stored at 4°C for not longer than 1 week.

Transmission Electron Microscopy (TEM)

SEV from human serum, HEK 293 cell culture supernatant or synovial fluid were purified and resuspended in PBS. A drop of purified EV was placed on parafilm and a formvar carbon coated nickel grid (Plano, Wetzlar, GER) was placed on top of the drop for 30–60 min. The samples were fixed with 2% paraformaldehyde (Carl Roth, Karlsruhe, GER) for 10 min and washed three times with MQ. SEV were examined using a Zeiss EM109 electron microscope.

FACS Analysis

Samples containing sEV were thawed in a water bath for ∼5 min at 37°C. After a short vortex, 20 µl of sample were incubated for 20 min in the dark with anti-CD63 FITC (abcam, Cambridge, UK). All samples were measured by flow cytometry on a Beckman Gallios instrument (Beckman Coulter, Brea, USA). Thresholds were set to side scatter in order to increase instrument sensitivity and measurements were performed for 60 s. SEV are presented as number of events positive for CD63, minus background noise which was determined using sEV-free buffers (PBS). Conjugate isotype-matched immunoglobulin (FITC) with no reactivity against human antigens was used as negative control.

Western Blotting

Twenty to thirty microgram of purified sEV were lysed in radioimmunoprecipitation assay buffer (RIPA), consisting of 50 mM Tris-Cl pH 7.4 (Sigma-Aldrich, Darmstadt, GER), 150 mM NaCl (Sigma-Aldrich, Darmstadt, GER), 1% NP40 (Sigma-Aldrich, Darmstadt, GER), 0.25% Na-deoxycholate (Sigma-Aldrich, Darmstadt, GER), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, Darmstadt, GER), EDTA-free protease inhibitor (Roche, Basel, CHE). SEV concentration was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Waltham, USA) with absorbance at 280 nm. Western blot analysis was performed according to (17). The membranes were incubated with primary antibodies that recognize CD63, CD9, CD81 and Heat shock protein 70 (Hsp70) (all purchased from System Biosciences, Palo Alto, USA). Membranes then were incubated with infrared dye conjugated secondary antibodies (IRDye®, LI-COR® Bioscience, Lincoln, USA) for 45 min at room temperature. Visualization was carried out using Odyssey Infrared Imaging System (LI-COR® Biosciences, Lincoln, USA).

Overexpression of miR-574-5p in sEV

The XMIRXpress Lenti system (System Biosciences, Palo Alto, USA) was used to overexpress the level of miR-574-5p in sEV (miR-574-5p oe). As negative control (XMIRXP-NT), which inherits a scrambled control, was also purchased by System Biosciences. Twenty-four hours prior to transfection HEK 293 cells were seeded at a density of 7×10^5 cells/well in a 6-well plate. Two microgram of either negative control or miR-574-5p oe plasmid were transiently transfected using Lipofectamine 2000®

(Invitrogen, Karlsruhe, GER). Supernatants were harvested for sEV isolation after 16 h. The overexpression efficiency was analyzed by RT-qPCR.

RNA Extraction

Total RNA from synovial fibroblasts was extracted using miRNeasy Mini Kit (Qiagen, Hilden, GER) according to the manufacturer's instructions. Residual DNA was removed by on-column DNAse digestion using RNase-Free DNase Set (Qiagen, Hilden, GER). Total RNA from purified sEV was isolated using the phenol/guanidinium thiocyanate (GTC)-based extraction method according to (18). 200 nM of a non-human synthetic cel-miR-39-3p (5′-UCACCGGGUGUAAAUCAGCUUG-3′, Sigma Aldrich, Darmstadt, GER) was added as internal standard to compensate for technical and methodical variations.

Real-time Quantitative PCR (RT-qPCR)

Intracellular and the extracellular miR quantification were performed using RT-qPCR analysis according to (14, 18). qRT-PCR was performed using the following primer: for miR-16-5p (MS0031493), miR-146a-5p (MS00003535), miR-155-5p (MS00031486), miR-574-5p (MS00043617, all from Qiagen, Hilden, GER). For intracellular miR quantification snRNA U6 was used as endogenous control primer (MS00033740, Qiagen, Hilden, GER) and as control for extracellular normalization we used cel-miR-39-3p (MS00019789, Qiagen, Hilden, GER). According to (14), different mRNA transcripts were analyzed. The following primer pairs were used: Interferon α (IFNα-fwd: 5′-GCAAGCCCAGAAGTATCTGC-3′; IFNα-rev: 5′-CTTGACTTGCAGCTGAGCAC-3′), Interleukin 23 (IL-23-fwd: 5′-GTTCCCCATATCCAGTGTGG-3′; IL-23-rev: 5′-AAAAATCAGACCCTGGTGGA-3′), TNFα (TNFα_fwd: 5′- CCCAGGGACCTCTCTCTAATC-3′; TNFα_rev: 5′- ATGGGCTACAGGCTTGTCACT-3′), IL-1β (IL1-β-fwd: 5′-ACAGATGAAGTGCTCCTTCCA-3′; IL1-β-rev: 5′-GTCGGAGATTCGTAGCTGGAT-3′) or IL-8 (IL-8-fwd: 5′-AGCTCTGTGTGAAGGTGCAG-3′; IL-8-rev; 5′-TGGGGTGGAAAGGTTTGGAG-3′). In all experiments β-Actin (Actin-fwd: 5′-CGGGACCTGACTGACTAC-3′; Actin-rev: 5′-CTTCTCCTTAATGTCACGCACG-3′) was used as endogenous control to normalize variations in cDNA quantities in different samples.

Live Cell Imaging

Purified sEV were labeled with lipophillic tracer 3,3′-Dioctadecyloxacarbocy-anine perchlorate (DiO, Sigma-Aldrich, Darmstadt, GER) for 15 min at 37°C and applied to $CD14^+$ monocytes or HeLa cells which were stained with 5 μg/ml Hoechst 33258 (Sigma-Aldrich, Darmstadt, GER) for 30 min. Pictures were taken every 10 min. Confocal images were acquired using an UltraVIEW VoX spinning disk system (PerkinElmer, Waltham, USA) mounted on a Nikon TI microscope (Nikon, Minato, Japan) and equipped with a climate chamber (37°C, 5% CO_2, 60% humidity). Images were taken at 1 μm Z increments and acquired with a cooled 14-bit EMCCD camera (1,000 × 1,000 pixel frame transfer EMCCD, 30 fps at full frame 1 × 1 binning 35 MHz readout, 8 × 8 μm pixel size) using Volocity 6.3 (PerkinElmer, Waltham, USA).

RNase and TritonX 100 Treatment of sEV

Isolated miR-574-5p oe sEV were treated either with 0.05 mg/ml RNase A (Qiagen, Hilden, GER) for 20 min at 37°C or with 1% TritonX 100 (Carl Roth, Karlsruhe, GER) for 10 min at RT and 0.05 mg/ml RNase A for 20 min at 37°C in combination. After RNA extraction, the amount of miR-574-5p was analyzed by RT-qPCR.

Osteoclast Differentiation

Monocytes were isolated from blood donor buffy coats using Ficoll-Paque™ Plus (GE Healthcare, Chicago, USA) separation and $CD14^+$ monocytes were selected using CD14 beads (MiltenyiBiotec, Bergisch-Gladbach, GER) according to the manufacturer's instructions. 1×10^5 cells/well were seeded in a 96-well plate in DMEM and differentiated into macrophages using 25 ng/ml macrophage colony-stimulating factor (M-CSF, Peprotech, Rocky Hill, USA) for 3 days. 50% of the culture medium was changed every 3 days and cells were further matured into OCs by medium supplements of 10 ng/mL M-CSF and 5 ng/mL Receptor Activator of NF-κB Ligand (RANKL, R&D Systems, Minneapolis, USA). The number of OCs was assessed using tartrate-resistant acid phosphatase (TRAP) staining (leucocyte acid phosphatase kit 387A, Sigma-Aldrich, Darmstadt, GER). Number of OCs was counted using a light microscope. $TRAP^+$ cells with at least or more than three nuclei were considered as being OCs. Each biological replicate has four technical replicates.

Stimulation of $CD14^+$ Monocytes

$CD14^+$ monocytes were isolated and seeded at a density of 1×10^5 cells/well in a 96-well plate and stimulated as indicated with purified EV or Resiquimod - R848 (Invivogen, San Diego, USA), diluted in Tris-EDTA (TE) buffer. Cells were additionally treated with 200 nM of the TLR7/8 inhibitor ODN 2088 Control (2087) (MiltenyiBiotec, Bergisch-Gladbach, GER), diluted in TE buffer, 30 min prior to stimulation with EV or R848 at indicated experiments. Furthermore, cells were treated either with 5 or 50 nM of synthetic miR-574-5p (Sigma-Aldrich, Darmstadt, Germany) together with or without 1 μg/ml Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, USA).

Cytokine mRNA Analysis

$CD14^+$ monocytes were isolated and seeded at a density of 1×10^6 cells/well in a 12-well plate and stimulated either with 1 μg/ml purified scrambled control or miR-574-5p oe sEV or 10 ng/ml R848 for 4 h. For experiments with the TLR7/8 inhibitor ODN 2088 Control (2087), cells were treated with 200 nM inhibitor 30 min prior to stimulation with sEV or R848 positive control. The mRNA level of each cytokine was analyzed by RT-qPCR.

Microscale Thermophoresis (MST)

The Cyanine5-labeled hsa-miR-574-5p (5′-UGAGUGUGUGUGUGUGAGUGUGU [Cyanine5]-3′,

Sigma-Aldrich, Darmstadt, GER) was adjusted to 100 nM with PBS (pH7.4, Thermo Fisher Scientific, Waltham, USA) supplemented with 0.05% Tween 20 (NanoTemper Technologies, München, GER). A series of 16 1:2 dilutions was prepared with recombinant human TLR8 (H00051311_G01, Abnova, Taiwan) using the protein storage buffer (25 mM Tris-HCl pH 8.2% Glycerin), producing ligand concentrations ranging from 20.4 pM to 668 nM. For the measurement, each ligand dilution was mixed with one volume of labeled hsa-miR-574-5p which leads to a final concentration of hsa-miR-574-5p of 50 nM and final ligand concentrations ranging from 10.4 pM to 334 nM. After 5 min incubation, the samples were loaded into Monolith NT.115 Standard Treated Capillaries (NanoTemper Technologies, München, GER). Instrument parameters were adjusted to 60% LED power and 40% MST power. A negative control was performed using Cy5-labeled hsa-miR-16 (5′-UAGCAGCACGUAAAUAUUGGCG[Cyanine5]-3′, Sigma-Aldrich, Darmstadt, GER) with a final concentration of 50 nM and a final TLR8 concentration ranging from 6.36 pM to 209 nM. Instrument parameters were adjusted to 20% LED power and 60% MST power. Data of three independently measurements were analyzed (MO.Affinity Analysis software version 2.1.3, NanoTemper Technologies, München, GER) using the signal from an MST-on time of 1.5 s.

Immunofluorescence

1×10^6 $CD14^+$ cells were seeded on poly-L-lysine (Sigma-Aldrich, Darmstadt, GER) coated glass coverslips (12 mm, Neolab, Heidelberg, GER) allowed to settle for 24 h. Cells were fixed with 4% formaldehyde (FA, Carl Roth, Karlsruhe, GER) for 10 min. After washing 3 times for 3 min with PBS, cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, Darmstadt, GER) in PBS for 10 min. Subsequently, cells were blocked with 4% BSA (Sigma-Aldrich, Darmstadt, GER) in PBS for 20 min. The primary antibodies against TLR7 (MA5-16247, Invitrogen, ThermoFisher Scientific, Waltham, USA) or TLR8 (PA5-20056, ThermoFisher Scientific, Waltham, USA) were diluted 1:50 (TLR7) or 1:100 (TLR8) in blocking solution and incubated for 1 h at room temperature. Afterwards, cells were washed with 0.01% Tween20 (Carl Roth, Karlsruhe, GER) in PBS 3 times for 5 min and incubated either with secondary antibody goat anti-mouse IgG (Alexa Fluor® 594, ab150116, abcam, Cambridge, UK) diluted 1:500 for TLR7 or goat anti rabbit IgG (Alexa Fluor® 488, 111-454-144, JacksonImmunoResearch, Ely, UK) in blocking solution for 45 min at room temperature. Finally, cells were washed with 0.01% Tween20 (Carl Roth, Karlsruhe, GER) in PBS and counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, Darmstadt, GER) for 5 min and mounted in 1,4-Diazabicyclo[2.2.2]octane (DABCO, Sigma-Aldrich, Darmstadt, GER). Fluorescence microscopy was carried out using a Zeiss Axiovert 200 microscope. Images were taken with Plan-Apochromat 100x/NA 1.4 (pixel size XY = 66 nm) oil immersion objective and a Zeiss AxioCam mRM camera.

Fuorescences *in-situ* Hybridization of miR-574-5p

2.5×10^5 $CD14^+$ monocytes were seeded on 12 mm glass coverslips in a 24-well plate and differentiated into M2-like macrophages by addition M-CSF to a final concentration of 25 ng/ml and further cultivation for 3 days.

Cells were fixed in formaldehyde (4% in PBS, 10 min at room temperature) followed by Trition X-100 permeabilization (0.5% in PBS, 10 min at room temperature), samples were washed three times with PBS containing 0.01 % Tween20, respectively. Coverslips where then placed at 37°C for 30 min covered with 1x miR ISH buffer (Qiagen, Hilden, GER, Cat No./ID: 339450) for prehybridization. Coverslips where then transferred into a hybridization chamber and covered with double DIG labeled anti miR-574-5p probe (Qiagen, Hilden, GER, 100 nM in 1x miR ISH buffer). Hybridization was carried out at 54°C for 1 h. Samples were transferred into a humidified staining chamber and washed twice with 54°C warm 2x SSC buffer (20x SSC stock solution, Invitrogen Karlsruhe, GER). Blocking was performed with 2% BSA (in PBS, 20 min at room temperature). Antigen detection was performed over night at 4°C. Anti-miR-574-5p DIG probe was detected by rabbit anti-DIG antibodies (Thermo Fisher Scientific, Waltham, USA, 9H27L19, 1 ng/ml final, diluted in blocking buffer) and TLR8 was detected by mouse anti-TLR8 antibodies (Thermo Fisher Scientific, Waltham, USA, 44C143, 1 ng/ml final, diluted in blocking buffer). Samples were washed three times with PBS containing 0.01% Tween20. Primary antibodies where detected using donkey anti-rabbit AlexaFluor 594 conjugated antibodies (abcam, Cambridge, UK, ab150080, 5 ng/ml final, diluted in blocking buffer) and donkey anti-mouse AlexaFluor 488 conjugated antibodies (abcam, Cambridge, UK, ab150105, 5 ng/ml final, diluted in blocking buffer) for 1 h at room temperature. Samples were washed again three times with PBS containing 0.01% Tween20. Nuclei where counterstained using DAPI (Sigma-Aldrich, Darmstadt, GER, 1 µg/ml, 10 min at room temperature). Samples were washed once with water and mounted using Mowiol (Sigma Aldrich, Darmstadt, GER).

Statistics

Results are presented as mean + SEM or ± SD. Statistical analysis was carried out using Student's unpaired *t*-test (two-tailed) or one-way ANOVA with turkey post-test using GraphPad Prism 6.0. Differences were considered as significant for $p < 0.05$ (indicated as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$). Dixon's test was performed as outlier test.

RESULTS

Isolation of sEV From Synovial Fluid of RA Patients

We isolated sEV from the synovial fluid of RA patients positive for anti-citrullinated protein antibodies ($ACPA^+$), which is associated with a more aggressive RA disease course and enhanced bone resorption (19). The vesicles were isolated using differential ultracentrifugation (**Supplementary Figure 1A**). The morphology and the size of the isolated vesicles were determined

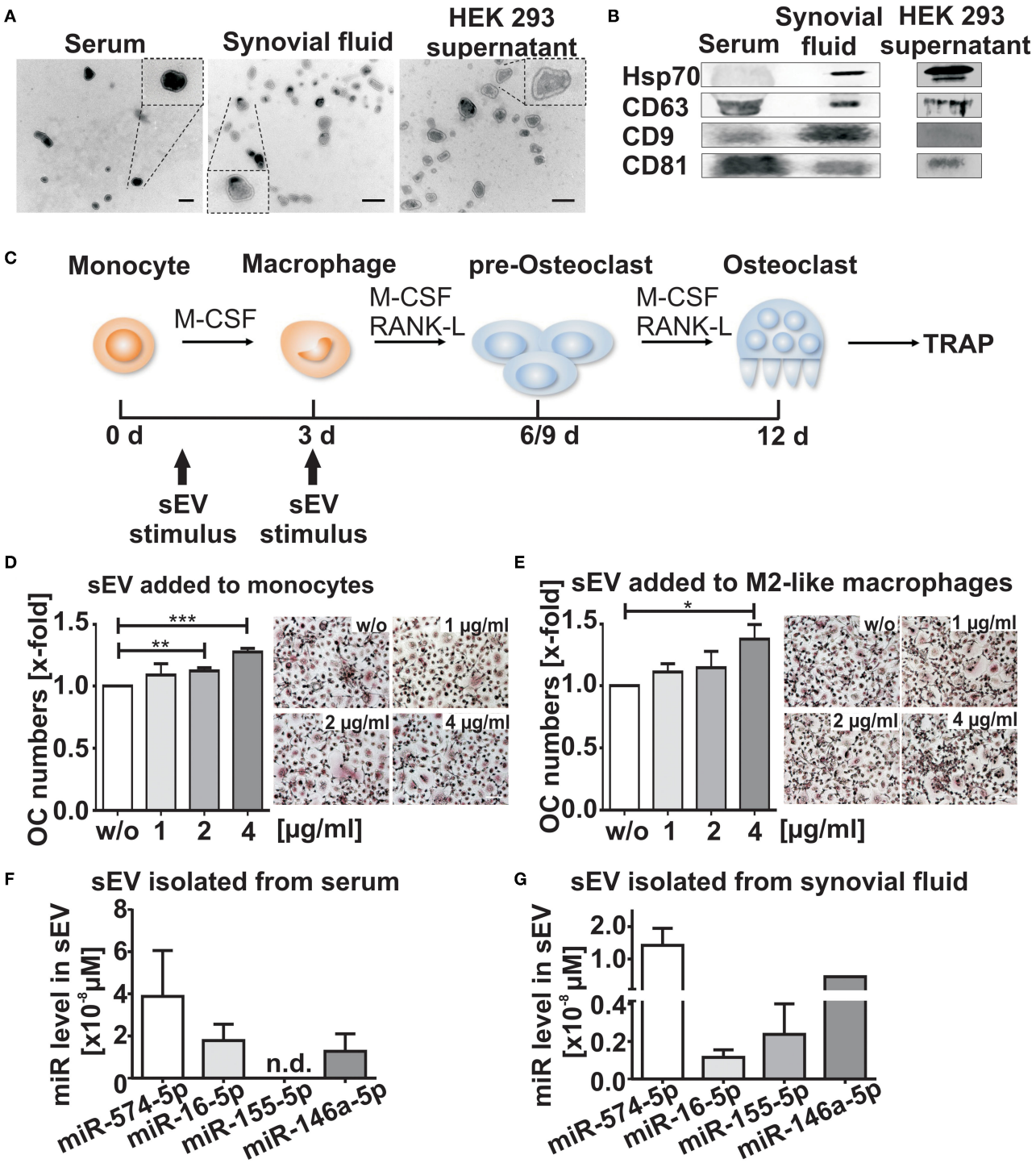

FIGURE 1 | Characterization of small extracellular vesicles (sEV). sEV were isolated by differential ultracentrifugation from serum, synovial fluid and HEK 293 cell supernatants. **(A)** Transmission electron microscopy (TEM), scale bar 200 nm. **(B)** Western blot analysis of CD9, Hsp70, CD63, and CD81 ($N = 3$). Blots and TEM pictures are shown from one representative experiment. **(C)** Schematic diagram of osteoclast (OC) differentiation. Cells were stimulated with sEV either at the monocyte stage (day 1) or the M2-like macrophage stage (day 3) as indicated by the arrows **(D,E)** Tartrate-resistant acid phosphatase (TRAP) staining of mature OCs obtained from $CD14^+$ monocytes and cultured in presence of sEV derived from synovial fluid of two $ACPA^+$ RA patients (1, 2, or 4 µg/ml). sEV were added to **(D)** $CD14^+$ monocytes and to **(E)** M2-like macrophages. Multinucleated cells with three or more nuclei that were stained with a purple color were considered as OCs. The relative changes are given as mean + SEM ($N = 4$), *t*-test $^{**}p < 0.01$, $^{***}p < 0.001$, scale bar 50 µm. Representative images of TRAP positive cells are shown. **(F,G)** sEV miR level of miR-574-5p,−16-5p,−155-5p, and−146a-5p isolated from 500 µl serum **(F)** or 500 µl synovial fluid **(G)** from three RA patients. Before sEV isolation, synovial fluid was pre-treated with hyaluronidase (1,500 U/ml) for 15 min at 37°C. MiR levels were determined by RT-qPCR and normalized to the spike-in control cel-miR-39-3p (200 nM). Data are shown as the mean + SEM ($N = 3$). $^*p < 0.05$.

by TEM, showing that the isolated population had the typical vesicular morphology and sEV size, ranging from 50 to 150 nm (**Figure 1A**). Using Western blotting, we were able to detect specific sEV surface protein markers such as CD63, CD9, Hsp70, and CD81 in sEV lysates (**Figure 1B**) (8).

sEV Derived From Synovial Fluid of RA Patients Induce Osteoclastogenesis

We next investigated whether sEV isolated from the synovial fluid of ACPA$^+$ RA patients influence osteoclastogenesis. CD14$^+$ monocytes were separated from peripheral blood mononuclear cells of healthy donors and stimulated with recombinant human M-CSF, RANKL (20) in combination with different concentrations of sEV (**Figure 1C**). In order to assess whether there were time- and maturation stage-related differences, both freshly isolated CD14$^+$ monocytes and M2-like macrophages (21) were treated with sEV (**Figures 1D,E**). After 9–12 days, cells were fixed and stained for the OC marker TRAP. We observed a significant dose-dependent increase (about 30%) in OC numbers, when the sEV were applied to monocytes (**Figure 1D**). A similar increase was observed, when macrophages, a later stage of OC differentiation, were treated with sEV (**Figure 1E**). These results suggest that the content of sEV derived from the synovial fluid of RA patients may contribute to an increased OC differentiation process.

Synovial Fluid Derived sEV Contain High Levels of miR-574-5p

It is known that miRs are selectively packaged in extracellular vesicles (22) and modulate inflammation and OC formation (23). Therefore, we selected four different miRs (hsa-miR-146a-5p, miR-155-5p, miR-16-5p and miR-574-5p) which previously have been associated with inflammation and/or osteoclastogenesis (14, 24–28). RT-qPCR was performed to analyze the level of each miR in sEV isolated from synovial fluid and in the corresponding serum samples from three RA patients. In contrast to miR-146a-5p, miR-16-5p and miR-155-5p, high levels of miR-574-5p were detected in sEV isolated from synovial fluid and serum samples.

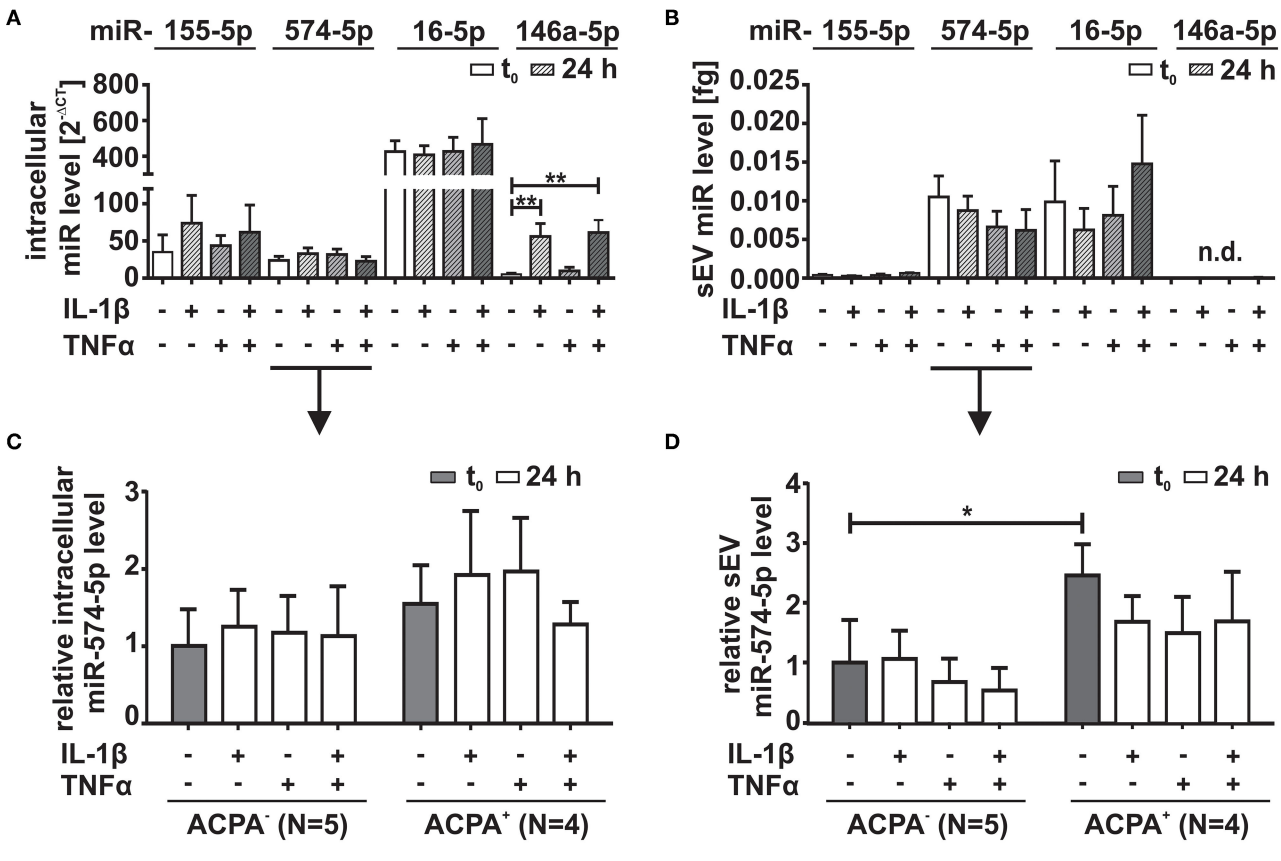

FIGURE 2 | Comparison of miR-574-5p, 16-5p, 155-5p, and 146a-5p levels in RA synovial fibroblasts (SFs). SFs from RA patients were cultured in sEV-depleted cell culture medium for 24 h. Medium was replaced by sEV-depleted cell culture medium supplemented with either 10 ng/ml IL-1β, 10 ng/ml TNFα or both for 24 h. MiR levels were determined by RT-qPCR. **(A)** Intracellular miR levels were normalized to snRNA U6 endogenous control. Data are shown as mean + SEM ($N = 10$), *t*-test $^{**}p < 0.01$. **(B)** MiR content in sEV derived from 1 ml cell supernatant was determined by RT-qPCR normalized to the spike-in control cel-miR-39-3p (200 nM). Data is shown as the mean + SEM of ($N = 9$). The results of **(C)** intracellular and **(D)** extracellular miR-574-5p were additionally evaluated with respect to patient characteristics. Therefore, miR-574-5p levels of SFs cells derived from ACPA$^-$ RA patients ($N = 5$) or RA patients ($N = 4$) were normalized to the mean of ACPA$^-$ patients. The relative changes are shown as the mean + SEM. $^*p < 0.05$.

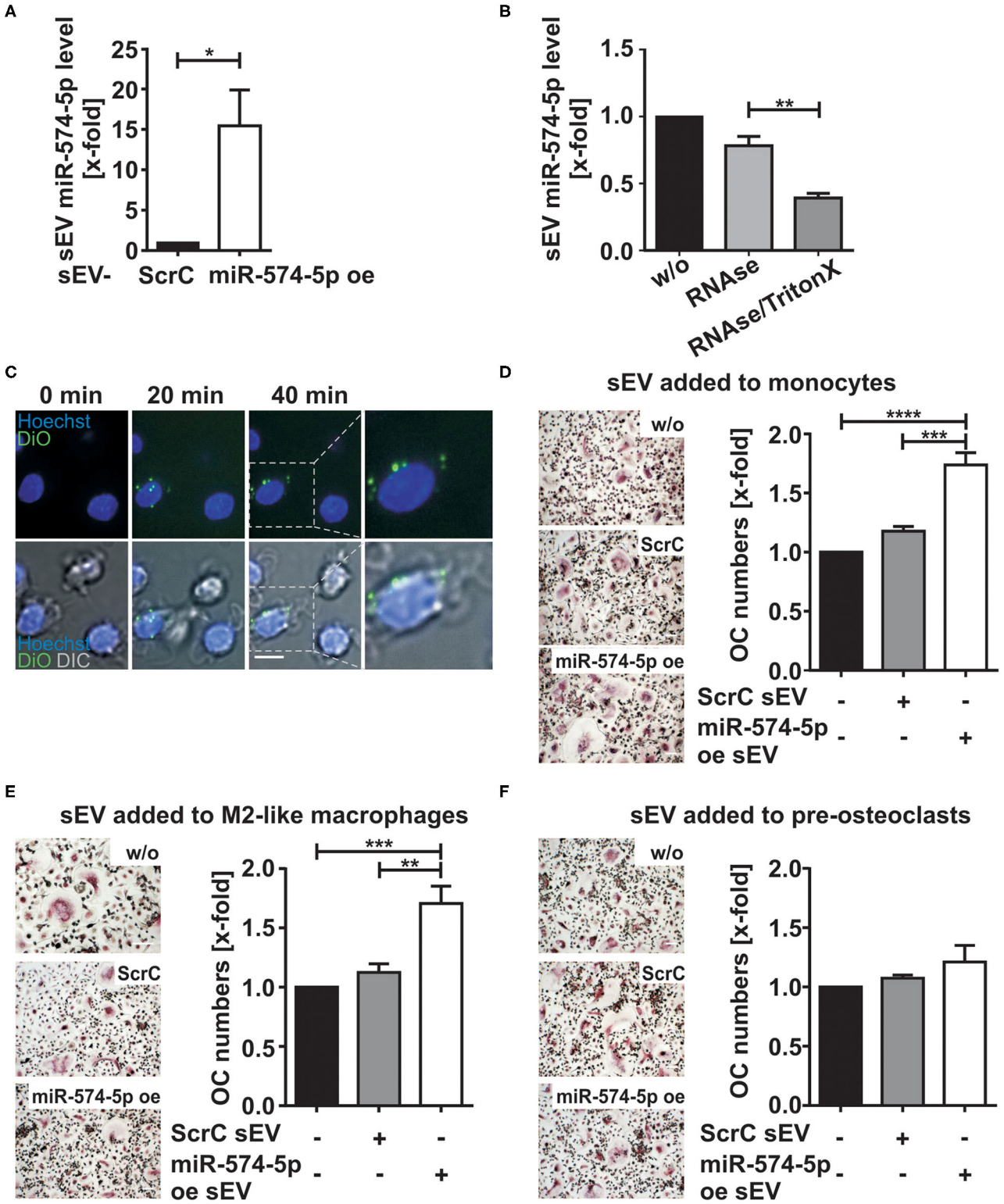

FIGURE 3 | Effect of sEV delivered miR-574-5p on OC differentiation. **(A)** HEK 293 cells were transfected with scrambled control (ScrC) or over expressed miR-574-5p (miR-574-5p oe) XMIRXpress construct. The sEV miR-574-5p level was analyzed by RT-qPCR and normalized to the spike-in control cel-miR-39-3p (200 nM). Relative changes to ScrC control are presented as the mean + SEM ($N = 4$), t-test $^*p < 0.05$. **(B)** miR-574-5p oe sEV were treated with 0.05 mg/ml RNase

(Continued)

FIGURE 3 | A with and without 1% TritonX. MiR-574-5p level was determined by RT-qPCR and normalized to the spike-in control cel-miR-39-3p. Relative changes to untreated sEV (w/o) are shown as the mean + SEM ($N = 3$), *t*-test $^{**}p < 0.01$. **(C)** Purified miR-574-5p oe sEV were labeled with lipophilic tracer 3,3′-dioctadecyloxacarbocy-anine perchlorate (DiO) and added to $CD14^+$ monocytes, which were stained with 5 μg/ml Hoechst 33258. Images were taken every 10 min, scale bar 10 μm. **(D–F)** TRAP staining of mature OCs obtained from $CD14^+$ monocytes. The cells were cultured in presence of 1 μg/ml ScrC or miR-574-5p oe sEV, which were added at **(D)** monocyte stage, **(E)** M2-like macrophage stage or **(F)** pre-osteoclast stage. TRAP-positive cells with three or more nuclei were considered as OCs. The relative changes normalized to untreated control cells are given as mean + SEM **(D,E)** ($N = 6$) and **(F)** ($N = 3$), one-way ANOVA $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.001$, scale bar 50 μm.

Notably, miR-146a was only detected once in sEV isolated from synovial fluid, while the same miR was consistently present in serum-derived sEV (**Figures 1F,G**).

Synovial Fibroblasts as Cellular Source of sEV Derived miR-574-5p

SFs play a crucial role in the pathogenesis of RA (29, 30). We therefore analyzed intracellular and corresponding sEV levels of miR-574-5p released from RA derived SFs using RT-qPCR and compared those to the levels of miR-146a-5p,−155-5p and−16-5p. In order to mimic the inflammatory environment of the inflamed joint, we stimulated SFs with 10 ng/ml IL-1β or 10 ng/ml TNFα alone or in combination for 24 h (**Figures 2A,B**). We observed that miR-155-5p, miR-16-5p, or miR-574-5p levels did not change significantly in response to IL-1β or TNFα stimulation. MiR-146a-5p was included in our experiments as a positive control. This miR has been reported to be strongly induced by IL-1β stimulation which our experiments also confirmed (31). TNFα stimulation alone had no influence on miR-146a-5p expression (**Figure 2A**). We next isolated sEV from SF cell culture supernatants and performed RT-qPCR. Very low levels of miR-155-5p or no miR-146a-5p were found in sEV regardless of stimulation. In contrast, high amounts of miR-16-5p and miR-574-5p were detected in the sEV purified from both cytokine stimulated and unstimulated SFs (**Figure 2B**), indicating that SFs are competent to secrete miR-574-5p containing sEV in RA joints. In concordance with a recent publication (32), we observed a slight but significant increase in the number of sEV in response to both IL-1β and TNFα stimulation (**Supplementary Figures 1B,C**).

Our next step was to compare intra- and extracellular miR-574-5p levels with regard to patients' ACPA status. No significant differences were evident comparing the intracellular expression of miR-574-5p in unstimulated SFs (**Figure 2C**). Contrary, higher concentrations of miR-574-5p were observed in sEV generated by SFs from $ACPA^+$ compared to $ACPA^-$ patients (**Figure 2D**).

However, the extracellular level of miR-574-5p was significantly higher in sEV derived from unstimulated SFs from $ACPA^+$ RA patients compared to SFs isolated from $ACPA^-$ patients (**Figure 2D**).

Overexpression of miR-574-5p in sEV

To assess whether the elevated miR-574-5p level in sEV had an influence on osteoclastogenesis, we established a miR overexpression system in HEK 293 cells that enhanced miR-574-5p loading into sEV (miR-574-5p oe sEV). For control experiments, we used sEV loaded with a scrambled miR (ScrC sEV). Comparing oe sEV with ScrC sEV (**Figure 3A**), we detected a ∼15-fold increase of miR-574-5p in the oe sEV. A RNase protection experiment was performed by treating the sEV with RNase alone or together with a detergent to determine if miR-574-5p was loaded into the sEV or if it was only attached to the sEV surface (33). The miR-574-5p was protected from RNase I digestion unless detergent was added to disrupt the membrane (**Figure 3B**). Therefore, we conclude that at least the majority of miR-574-5p is selectively loaded into the oe sEV. Finally, we could show that these sEV are taken up by cells like monocytes (**Figure 3C**) and HeLa cells (**Supplementary Figure 1D**), using confocal microscopic live cell imaging.

sEV With High miR-574-5p Levels Induce Osteoclastogenesis

In order to investigate the role of sEV derived miR-574-5p during osteoclastogenesis, we added either 1 μg/ml miR-574-5p oe sEV or ScrC sEV at different time points during OC differentiation (**Figures 3D–F**). After ∼12 days of differentiation, the cells were TRAP-stained and the number of OCs was counted.

When sEV were added at the stage of monocytes, a significant upregulation of OC numbers was observed in response to miR-574-5p oe sEV compared to ScrC sEV or untreated control (**Figure 3D**). We observed comparable results, when the engineered sEV were added at the stage of macrophages (**Figure 3E**). No significant changes in OC numbers were found when sEV were added to pre-OCs, neither with the addition of control or miR-574-5p oe sEV (**Figure 3F**). These results suggest that the effect of extracellular miR-574-5p strongly depends on the maturation stage of the cells during OC differentiation.

Increased Osteoclast Differentiation by miR-574-5p Is Mediated by TLR7/8

First hints indicating that miR-574-5p represents a novel TLR7/8 agonist (34) were based on its sequence homology to RNA33, a well-known TLR7/8 ligand (35) (**Figure 4A**). To validate a direct interaction between miR-574-5p and TLR8, MST (36) was performed using Cy5-labeled miR-574-5p and commercially available human recombinant TLR8. We observed strong binding of miR-574-5p to TLR8 with a dissociation constant (K_D) of 30.8 ± 5.24 nM (**Figures 4B,C**). As negative control we used miR-16-5p, a randomly chosen natural miR that contains no binding sequence for TLR8 (**Figure 4A**), which shows no binding capacity in MST analysis (**Figures 4B,C**).

We next investigated whether the increased OC differentiation was due to the activation of TLR7/8. During OC differentiation, all cell types were stained positively for TLR7/8 expression (**Supplementary Figures 2A,B**). Furthermore, occasional colocalization between miR-574-5p and TLR8 was observed in M2-like macrophages (**Supplementary Figures 2C,D**). Therefore, we isolated and stimulated monocytes and

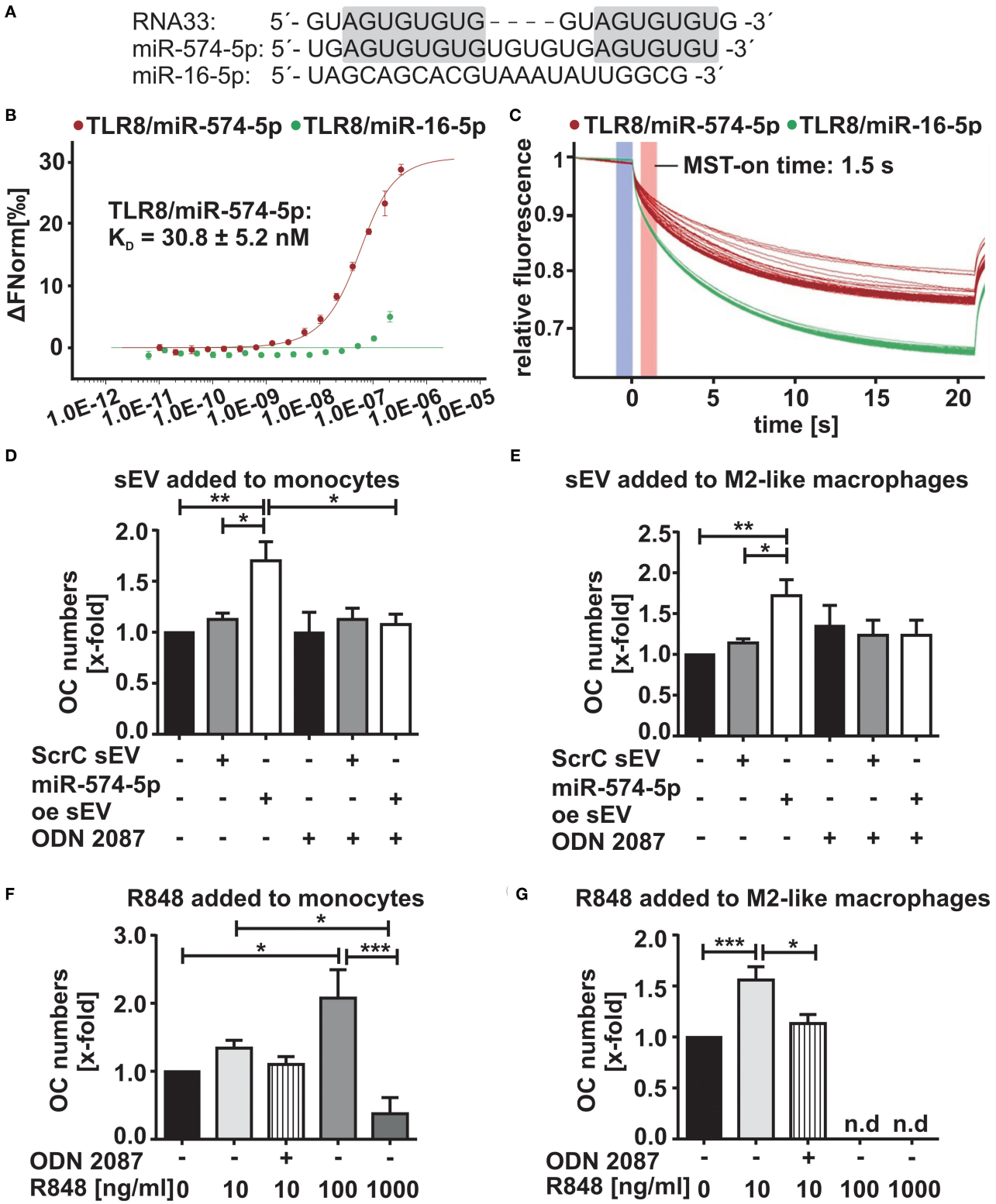

FIGURE 4 | Increased OC differentiation by sEV-delivered miR-574-5p is mediated by TLR7/8 activation **(A)** Comparison of sequence homologies of miR-574-5p and miR-16-5p to RNA33. **(B,C)** In microscale thermophoresis (MST) affinity assay of miR-574-5p to human recombinant TLR8, 50 nM of Cy5-labeled miR-574-5p and 334 nM−10.2 pM of non-labeled TLR8 were used. As negative control, 50 nM of Cy5-labeled hsa-miR-16-5p with 209 nM−6.36 pM of TLR8 were used. After a short

(Continued)

FIGURE 4 | incubation, samples were loaded into Monolith NT.115 Standard Treated Capillaries and the MST measurement was performed **(B)** Dose response curve reveals a K_D of 30.8 ± 5.24 nM for the interaction of miR-574-5p and TLR8. Data are shown as ± SD ($N = 3$). **(C)** MST traces shown for miR-574-5p or miR-16-5p and TLR8. An MST-on time of 1.5 s was used for analysis. **(D–G)** TRAP-positive OCs obtained from $CD14^+$ monocytes cultured in presence of **(D,E)** 1 μg/ml ScrC- or miR-574-5p oe sEV together with 200 nM TLR7/8 inhibitor ODN 2087 or **(F,G)** 10–1,000 ng/ml - TLR7/8 ligand R848 added either at (D/F) monocyte stage or (E/G) M2-like macrophage stage. TRAP-positive cells with three or more nuclei were considered as OCs. The relative changes normalized to untreated control cells are given as mean + SEM ($N = 4$), one-way ANOVA, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

macrophages with miR-574-5p oe sEV or ScrC sEV together with 200 nM of the TLR7/8 inhibitor ODN 2087. As expected, the miR-574-5p-mediated effect was completely blocked by the addition of the inhibitor (**Figures 4D,E**; **Supplementary Figures 3A,B**). As a control experiment we used R848, a known TLR7/8 ligand (37, 38), which was added to the monocytes and macrophages instead of sEV at a concentration of 10 ng/ml in the same experimental setup. As previously with miR-574-5p oe sEV, a significant increase of OC numbers was observed (**Figures 4F,G**; **Supplementary Figures 3C,D**) that was blocked using TLR7/8 inhibitor. No increase in osteoclastogenesis was observed, when pre-OCs were stimulated with the TLR7/8 agonist (**Supplementary Figure 3E**). This result was consistent with the results with miR-574-5p oe sEV or ScrC sEV (**Figure 3F**). Notably, we observed an increase in OC differentiation, when monocytes or macrophages were stimulated with the TLR7/8 ligand R4848. While the agonist led to an increase in OC numbers at low doses, the opposite effect was observed at higher R848 concentrations. The number of OCs was drastically reduced, when cells were treated with 1 μg/ml R848 at all differentiation time points. The same negative effect was observed when 100 ng/ml of R848 were added to macrophages and pre-OCs (**Figures 4F,G**; **Supplementary Figure 3E**). In summary, our results strongly suggest that the increase in OC differentiation by sEV derived miR-574-5p is mediated by TLR7/8 activation.

sEV Delivered miR-574-5p Induces IFNα and IL-23 mRNA in CD14+-monocytes via TLR7/8 Activation

We next aimed to understand which cytokines were increased by sEV with high miR-574-5p levels. Therefore, we stimulated $CD14^+$ monocytes either with 4 μg/ml of sEV isolated from the synovial fluid of $ACPA^+$ RA patients or with 1 μg/ml of miR-574-5p oe sEV or ScrC sEV for 4 h. Total RNA was isolated and the mRNA levels of different TLR7/8 target genes such as IL-23, IL-8, INFα, IL-1β, and TNFα were analyzed (**Figure 5**; **Supplementary Figure 4**). These cytokines are known to influence OC differentiation (39). We observed no changes in IL-23 mRNA levels, while IFNα mRNA levels increased about ∼3-fold, when cells were treated with sEV isolated from synovial fluid (**Figures 5A,B**). When stimulating the monocytes with miR-574-5p oe sEV, we observed significant 2- and 5-fold inductions of IL-23 and IFNα mRNA, respectively, while ScrC sEV had no effect (**Figures 5C,D**). The induction of IL-23 and IFNα was reversed by additional application of the TLR7/8 inhibitor. Comparable results were obtained when monocytes were stimulated with 10 ng/ml of the TLR7/8 ligand R848 (**Figures 5E,F**). In contrast to IL-23 and IFNα, the mRNA levels of TNFα, IL-1β, and IL-8 were not significantly affected by sEV isolated from the synovial fluid of RA patients (**Supplementary Figures 4A–C**), miR-574-5p oe sEV or R848 (**Supplementary Figures 4D–I**).

DISCUSSION

sEV were identified as a key factor in cell-to-cell communication through transfer of miRs (10, 22). A better understanding of their physiological function in the synovial microenvironment of RA patients is essential for the development of novel treatment strategies. While previous studies have shown that extracellular vesicles play an important role in the pathogenesis of RA (40), the molecular mechanism and their impact on bone resorption needs to be further elucidated. Our study shows for the first time that sEV isolated from synovial fluid of RA patients caused an increased OC differentiation, which we attribute to high abundance of miR-574-5p in the sEV.

Different cell types in the joint microenvironment can contribute to aggressive cartilage and bone resorption (1). In particular, activated SFs strongly induce osteoclast formation (29, 30). We identified SFs as a cellular source of sEV with high miR-574-5p content. Despite the low number of patient-derived synovial fibroblasts, we observed higher levels of miR-574-5p in the sEV derived from $ACPA^+$ compared to $ACPA^-$ RA patients. Since ACPA positivity is associated with a more severe and aggressive course of RA (19, 41), it is intriguing to propose that miR-574-5p might play a role in $ACPA^+$ RA. However, it is for future studies to identify the source of high miR-574-5p levels in $ACPA^+$ RA patients and to investigate the potential impact of ACPAs on miR-574-5p secretion into sEV.

In order to perform functional assays, we established an overexpression system in which sEV were loaded with high amounts of miR-574-5p. We demonstrated that miR-574-5p oe sEV were taken up by cells and were physiologically active. $CD14^+$ monocytes and M2-like macrophages were stimulated with miR-574-5p oe sEV and significant changes in OC formation were observed compared to controls. No changes were observed when the same sEV were applied to pre-OCs, indicating that only a certain progenitor cell stage is responsive to extracellular miR-574-5p. Although all cell types show TLR7/8 expression, it can be speculated that certain downstream proteins are induced in pre-OCs which negatively regulate TLR signaling in response to miR-574-5p stimulation (42).

Furthermore, it suggests that the sEV delivered miR-574-5p might have immune-modulating functions. miRs have proven to be regulators in immune response (43) via binding to TLRs (15, 44, 45). sEV-delivered miR-let-7b is able to transform human- and mouse naive monocytes into inflammatory M1-like macrophages by activating TLR7 (44). This highlights the

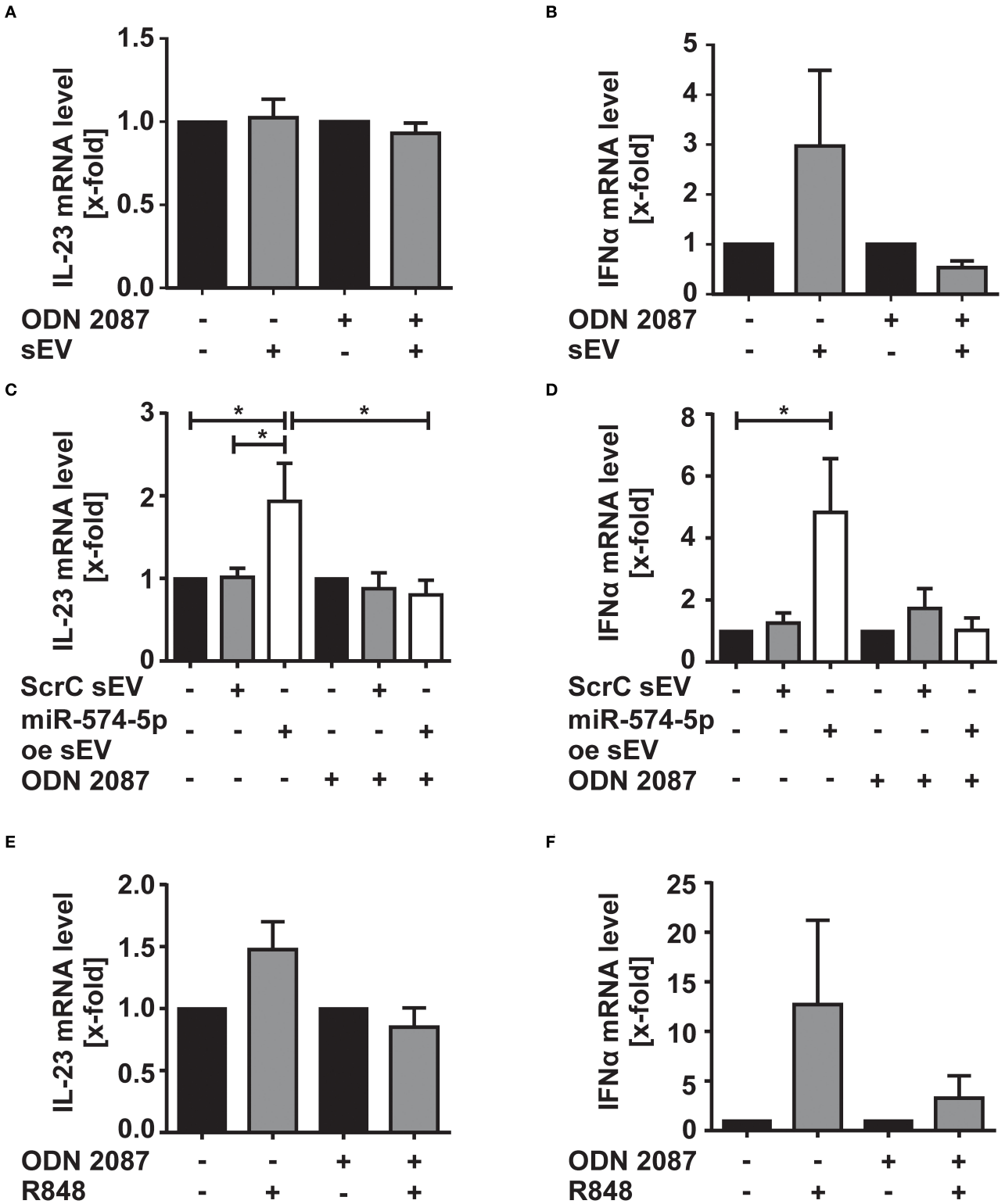

FIGURE 5 | Effect of sEV-delivered miR-574-5p on IL-23 and IFNα mRNA levels. CD-14+ monocytes were stimulated with **(A,B)** 4 μg/ml sEV isolated from synovial fluid of ACPA+ RA patients or **(C,D)** 1 μg/ml of ScrC or miR-574-5p oe sEV and 200 nM ODN 2087. Cells were harvested after 4 h of incubation and total RNA was extracted. Quantification of **(A,C)** IL-23 and **(B,D)** IFNα mRNA levels using RT-qPCR. **(E,F)** CD14+ monocytes were stimulated with 10 ng/μl R848 and 200 nM of ODN 2087 for 4 h. Total RNA was extracted, and RT-qPCR was performed to quantify **(E)** IL-23 and **(F)** IFNα mRNA level. β-Actin was used as endogenous control. Relative changes normalized to untreated controls are given as + SEM ($N = 4$), one-way ANOVA, $^*p < 0.05$.

importance of extracellular miRs in cell-to-cell communication and their impact on chronic inflammatory diseases. However, the mechanisms of extracellular miR release and their effects on target cells are not fully understood.

In our experimental setup, we demonstrated that miR-574-5p induces osteoclastogenesis only when loaded into sEV. Synthetic liposomal vehicles or synthetic miRs alone had no effect on osteoclastogenesis (**Supplementary Figures 5A,B**). These findings are in agreement with current understanding that vesicle-associated miRs are important players in the cell-to-cell communication, while free miRs may only represent cell by-products without physiological impact. We can speculate that sEV uptake depends on proteins and glycoproteins expressed on the surface of the vesicle as well as on the surface of the target cell (46). Furthermore, Fabbri et al., demonstrated that extracellular vesicle-delivered miR-21 and−29a bind to human TLR8 and trigger downstream to NF-κB activation in the context of non-small cell lung cancer (15). Since miR-574-5p has a high sequence analogy to the TLR7/8 ligand RNA33 and induced phosphorylation of the p65 subunits of NF-κB (15, 34, 35), we asked whether miR-574-5p might act as direct TLR7/8 ligand and increases OC differentiation by TLR7/8 activation. With the results of our MST binding test and our experiments using the TLR7/8 inhibitor ODN 2087 and the agonist R848, we could show that miR-574-5p binds to the receptor and thus mediates increased OC formation via TLR7/8. However, our results seem to contradict a previous study by Miyamoto et al., which reports a decrease in OC formation in response to R848 stimulation (47). This inconsistency could be explained by slight experimental differences such as the R848 concentration and the cell types used to analyze the OC maturation process. Our results indicate that sEV delivered miR-574-5p has the highest influence on osteoclastogenesis at monocyte and M2-like macrophage stages compared to the pre-OC stage. This is probably due to the binding of miR-574-5p to TLR7/8 whose expression level is reduced during the monocyte differentiation process (48). In addition, our results are supported by findings of Salvi et al., who showed that extracellular miR-574-5p can promote production of IFNα by inducing human TLR7 activation in human plasmacytoid dendritic cells (34). In fact, we observed similar results when we treated monocytes with miR-574-5p oe sEV stimulation or TLR7/8 ligand R848. In both cases, we observed an increase in IFNα and IL-23 mRNA levels. Extracellular miR-574-5p can therefore be considered as a new immune-modulating mediator which strongly influences bone resorption in RA via its function as TLR7/8 ligand.

Additionally, it has recently been shown that an elevated intracellular miR-574-5p expression is directly associated with an enhanced synthesis of prostaglandin E_2 (14), an important pro-inflammatory lipid mediator which mediates inflammation in RA (49). This newly discovered link between miR-574-5p, inflammation and OC-mediated bone resorption offers the opportunity to develop new RNA-therapeutics. Inhibitors against miR-574-5p would address simultaneously its intracellular function as a regulator of prostaglandin synthesis and its endosomal function as TLR7/8 ligand, which would inhibit bone resorption in arthritis disease such as RA.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**supplementary material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Ethical Committee (Solna, Stockholm, Sweden, the ethical permit number 2009/1262-31/3) and is in compliance with all ethical standards and patients' consent according to the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AH performed the biochemistry experiments, analyzed the data, and wrote the manuscript. KB performed the colocalization staining, supported the experiments, and contributed to writing. SO performed the MST analysis and supported the experiments. FM performed the FACS analysis. MK, P-JJ, AC, and HW provided patient material. BR and HW designed and supported the experiments. HW contributed to writing. MS conceived the study, designed and supervised the overall project, and wrote the manuscript. All authors conducted the quality assurance of the paper and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.585282/full#supplementary-material

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Conflict of Interest: MS and AH have submitted a patent application (DE 102019122014.9) "Inhibition of miR-574-5p as novel therapeutic strategy to reduce bone resorption in arthritis disease."

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Emerging Roles of Exosomes in T1DM

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Type 1 diabetes mellitus (T1DM) is a complex autoimmune disorder that mainly affects children and adolescents. The elevated blood glucose level of patients with T1DM results from absolute insulin deficiency and leads to hyperglycemia and the development of life-threatening diabetic complications. Although great efforts have been made to elucidate the pathogenesis of this disease, the precise underlying mechanisms are still obscure. Emerging evidence indicates that small extracellular vesicles, namely, exosomes, take part in intercellular communication and regulate interorgan crosstalk. More importantly, many findings suggest that exosomes and their cargo are associated with the development of T1DM. Therefore, a deeper understanding of exosomes is beneficial for further elucidating the pathogenic process of T1DM. Exosomes are promising biomarkers for evaluating the risk of developingty T1DM, monitoring the disease state and predicting related complications because their number and composition can reflect the status of their parent cells. Additionally, since exosomes are natural carriers of functional proteins, RNA and DNA, they can be used as therapeutic tools to deliver these molecules and drugs. In this review, we briefly introduce the current understanding of exosomes. Next, we focus on the relationship between exosomes and T1DM from three perspectives, i.e., the pathogenic role of exosomes in T1DM, exosomes as novel biomarkers of T1DM and exosomes as therapeutic tools for T1DM.

Keywords: type 1 diabetes mellitus, exosomes, biomarkers, microRNAs, therapy

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INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder characterized by beta-cell dysfunction and death caused by autoreactive T cells, an absolute lack of insulin, and elevated blood glucose levels (1–3). Persistent hyperglycemia leads to the development of life-threatening diabetes-associated complications such as blindness, stroke, kidney diseases, and heart diseases, thus decreasing the quality of life of patients and imposing a considerable economic burden on society and individuals.

Currently, it is widely accepted that a combination of genetic and environmental factors contribute to an increased risk of T1DM (4–6). Although substantial research efforts have been made to elucidate the pathophysiology of T1DM, the exact underlying mechanisms are still largely

unknown. For example, the critical initial triggering events that result in infiltration of T lymphocytes and pancreatic islet autoimmunity, which are important in early identification of T1DM and effective prevention of further islet deterioration, have not been revealed. Because the pathogenic mechanisms are obscure, most patients with T1DM rely on life-long exogenous insulin administration, which only alleviates symptoms. Today, the most commonly used biomarkers of T1DM are human leukocyte antigen (HLA) genes and islet autoantibodies. However, these biomarkers do not fully meet current needs. The ideal biomarkers should be objective indicators of disease condition that can be measured accurately and reproducibly, should identify disease stage and progression and should assess the outcome of therapies.

Exosomes, which are small vesicles carrying bioactive molecules such as DNA, RNA, and proteins, have emerged as important mediators of cellular and interorgan communication. Evidence shows that exosomes may be involved in the loss of tolerance towards islet cells and take part in islet autoimmunity (7). Therefore, a better understanding of exosomes may provide novel insight into the onset and development of T1DM. In addition, studies have indicated that the number and composition of exosomes can reflect the physical and pathological status of their cells of origin, which means that monitoring exosomes can be helpful for disease diagnosis. Therapeutically, exosomes have the potential to be exploited as novel treatment agents and drug delivery vectors. Therefore, more comprehensive knowledge of exosomes may not only help reveal the underlying pathogenic mechanisms of T1DM but also provide valuable targets for use as disease biomarkers and therapeutic tools.

EXTRACELLULAR VESICLES AND EXOSOMES

In recent years, in addition to cytokines, chemokines and hormones, a new group of modulators called extracellular vesicles (EVs) that can regulate cell-to-cell communication have emerged (8). EVs are a group of heterogeneous lipid bilayer-enclosed structures that are secreted into the extracellular milieu by multiple types of cells. These small membrane-bound structures can be released by almost all cell types in response to endogenous and exogenous stimulation (9). According to their biogenesis, size, content and biological function, EVs can be mainly classified as exosomes (EXOs), apoptotic bodies and microvesicles (MVs) (10–12). However, given the consensus has not been reached on specific markers of EV subtypes and the fact that distinguishing the biogenesis pathway of EVs remains extremely difficult, MISEV 2018 (Minimal information for studies of extracellular vesicles 2018) recommended authors to use operational terms for EV subtypes that refer to physical characteristics of EVs, biochemical compositions, and description of conditions or cell of origin (13). But in this review, we adopt the description used by reference literature for the sake of convenience in the recital.

Exosomes, which range from 30 to 200 nm in diameter, are present in various kinds of biological fluids, such as serum, cerebral spinal fluid, saliva, urine, pleural effusion or ascites, and breast milk (14–16). They can mediate intercellular communication *via* cargo molecules. The cargo delivered by EXOs includes DNA, RNA (miRNA, tRNA, mRNA, rRNA), proteins and lipids (8, 17). Because the cargo of EXOs can reflect the status of their cells of origin, monitoring and repurposing these nanovesicles can be useful for the diagnosis and therapy of many diseases, including type 1 diabetes mellitus (T1DM). EXOs are formed through endosomal networks, and they thus bear specific markers such as tetraspanins (CD9, CD63, and CD81), heat shock proteins (HSP70), and the Rab family proteins Tsg101 and Alix (18, 19). The biogenesis of EXOs can be divided into three stages: (1) the invagination of early endosomes, which engulfs content from the cytoplasm; (2) the formation of multivesicular bodies (MVBs) *via* inward budding of the endosomal membrane; and (3) the fusion of MVBs with the plasma membrane and secretion of exosomes (20). The biogenesis of EXOs is strictly regulated by multiple factors, such as the cell type (14), contact inhibition (21, 22), cell culture (23), Ca^{2+} (24), and hypoxia (25). More importantly, many pathological states, such as cancer (26, 27), diabetes (28), and neuronal degradation (29, 30), affect the yield and content of exosomes, making it theoretically feasible to apply exosomes for the diagnosis and treatment of diseases. Upon release, these exosomes can induce biological responses of recipient cells *via* a range of processes, including protein-protein interactions on the cell surface or entry into the cytosol of recipient cells through endocytosis and fusion with the plasma membrane (28). Compared with exosomes, the biogenesis of the MVs (50–2,000 nm in size) is far less learned. In general, the formation of MVs is resulted from dynamic interplay between phospholipid redistribution and cytoskeletal protein contraction, which is distinct from the biogenesis of exosomes (31). Also, the cargo of MVs tends to be highly enriched for specific proteins which are different from exosomes. For example, a study indicated that the MVs secreted by melanoma cells are enriched for B1 intergrin receptors (32). On the other hand, transferrin receptors, which are highly detected in exosomes, are missing in MVs (33). Unlike exosomes and MVs are produced during normal cellular process, the formation of apoptotic bodies (500–4000 nm in size) is associated with programmed cell death. This process is characterized by condensation of the nuclear chromatin, membrane blebbing, and the cellular content enclosed by apoptotic bodies (34). Most apoptotic bodies will be eliminated by macrophages locally (35).

Given the emerging roles of exosomes in multiple physiological and pathogenic processes, extensive effort has been applied to further understand exosomes and improve their isolation methods. A variety of technologies, including ultracentrifugation, affinity-based capture technology, filtration, chromatography, precipitation, and microfluidics, have been developed to isolate EXOs. However, given that the size of EXOs is extremely small, isolation is very challenging, and all these techniques have their own limitations (14). Therefore, it is

imperative to develop or advance new or existing methods to isolate exosomes.

THE PATHOGENESIS OF T1DM

The pathogenesis of T1DM is associated with a complex interplay between genetic and environmental factors. However, the early triggering events of T1DM are still largely unknown. It is of particular importance to elucidate the factors that lead to beta-cell-specific T cell intolerance; these factors may include genetics, exogenous infection, endogenous superantigens, physiological stress events, and noninfectious environmental elements (36). In nonobese diabetic (NOD) mice, an excellent animal model representing human T1DM, the breakdown of tolerance to pancreatic islet self-antigens occurs spontaneously in early life. Before lymphocyte infiltration, physiological abnormalities of the islets, including vascular pathology, increased endoplasmic reticulum (ER) stress, and enhanced expression of inflammatory cytokines, are present in the pancreas in NOD mice (37, 38). These events may lead to beta-cell dysfunction and death, thus leading to the release of autoantigens and the activation of specific autoreactive T cells.

In addition, some reports show that stromal cells, rather than endocrine cells, might be critical factors inducing local inflammatory responses and subsequent islet autoimmunity. For example, peri-islet Schwann cells have been proposed as early targets involved in the initial peri-insulitis, and a specific population of T cells targeting Schwann cell antigens has been identified (39, 40). However, given that these cells do not express candidate antigens of T1DM and some lymphocyte-infiltrated islets do not undergo the peri-insulitis stage, it can be speculated that Schwann cells are not the only contributor. Additionally, islet endothelial cells (IECs) are associated with early triggering events of T1DM because they may facilitate the infiltration of autoreactive T cells into islets (41). Moreover, it has been reported that lymphatic vessel endothelial cells are also involved in islet inflammatory responses (42). It is more likely that both beta-cells and stromal cells contribute to early triggering events. Interestingly, some findings have indicated that exosomes can mediate communication between different cell types within the islets and have immunostimulatory as well as immunomodulatory properties, suggesting that they might serve as early agents inducing the initial events of T1DM (43).

THE POTENTIAL ROLE OF EXOSOMES IN T1DM

Emerging evidence has indicated that exosomes, which possess immunoregulatory functions, may participate in the initiation and development of autoimmune diabetes (**Figure 1**) (28, 44). On the one hand, islet-derived exosomes can activate the immune system and lead to autoimmune responses (**Table 1**) (53). At present, the exact mechanisms by which intracellular autoantigens are initially detected by the immune system and

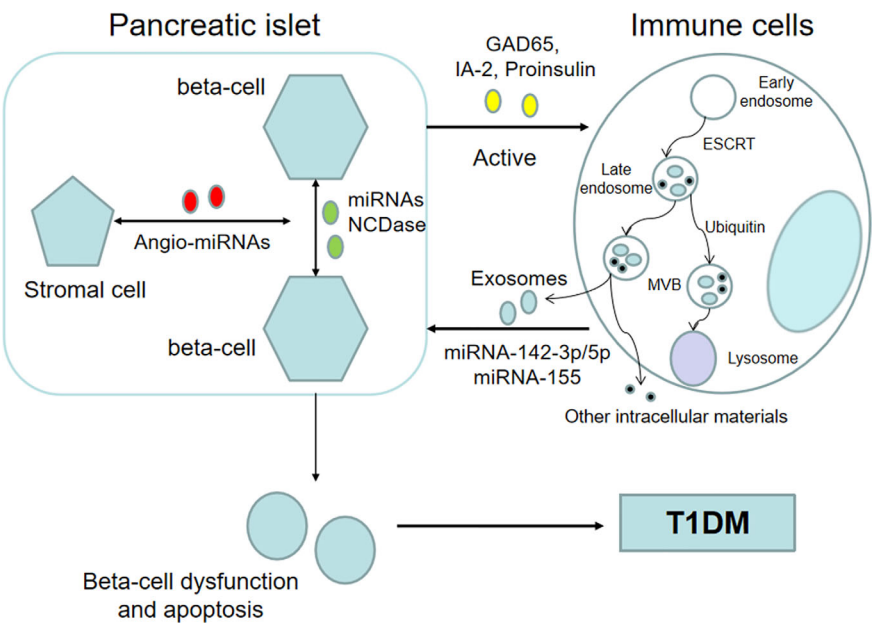

FIGURE 1 | Exosomes participate in the pathological process of T1DM. Beta cell-derived exosomes that contain islet autoantigens and specific miRNAs can activate the immune system. In return, immune cell-derived exosomes can induce beta-cell dysfunction and apoptosis, eventually leading to T1DM. In addition, exosomes can deliver biological information between beta-cells, and horizontal message transfer can coordinate beta-cell activity. Additionally, some studies have shown that exosomes may serve as mediators between insulin-producing beta cells and stromal cells and are associated with the revascularization process after islet transplantation.

TABLE 1 | Summary of findings on exosomes and T1DM.

Experimental subjects	Findings	References
Rats and humans	Exosomes released from the pancreatic islets contain beta-cell autoantigens and can activate adaptive immune responses	(45)
MIN6B1 cells	Exosomal miRNA transfer regulates the activity of beta-cells and transduces apoptotic signals	(46)
NOD mice	Exosomes released from islet-derived MSCs can trigger autoimmune responses in NOD mice	(7)
INS-1 cells	NCDase-containing exosomes released by INS-1 cells inhibit beta-cell apoptosis induced by high levels of inflammatory cytokines	(47)
MIN6 and NOD mice	Exosomes containing miR-29b released from beta-cells modulate innate and adaptive immune responses	(48)
NHI6F Tu28	Pancreatic beta-cells shed membrane-derived microvesicles	(49)
MIN6 and NOD mice	Insulinoma-released exosomes can activate autoreactive T cells in NOD mice	(50)
MIN6 and NOD mice	Insulinoma-released exosomes can activate autoreactive marginal zone-like B cells in prediabetic NOD mice	(51)
Human islets and NOD mice	Exosomal miRNAs derived from T lymphocytes promote pancreatic beta-cell death	(52)

presented to autoimmune T cells have not been fully elucidated in the pathogenesis of T1DM. Intriguingly, a recent finding indicates that rat and human pancreatic islets can release exosomes containing beta-cell autoantigens that belong to intracellular membrane proteins, including glutamic acid decarboxylase 65 (GAD65), islet-associated protein 2 (IA-2) and proinsulin, and released exosomes can be taken up by dendritic cells and lead to cell activation (45). Moreover, the anchoring of GAD65 to exosome-mimetic liposomes, whose size and lipid composition is similar to islet exosomes, can enhance antigen presentation and T cell activation in individuals susceptible to T1DM (45). In addition, a previous study indicated that mouse MIN6 insulinoma cells can release exosomes that express GAD65 (50). These studies indicated that exosomes might be important agents in the development of T1DM. However, there are some problems remained to be clarified. For example, because the findings are drawn based on *in vitro* experiments, *in vivo* studies are necessary. In addition, apoptotic beta-cells can also release autoantigens which can be taken by APCs in pancreatic lymph nodes and activate autoimmune responses. So whether exosomes are primary drivers in the initiation of autoimmune responses against pancreatic beta cells or rather are secondary contributors in the development of T1DM needs further investigation. But given the secretion of exosomes is a positive process and can occur before beta-cell destruction, they are seemed to play more critical role in the initiation of autoimmune responses.

Moreover, exosomes are closely associated with physiological islet abnormalities prior to lymphocytic infiltration, including increased ER stress in beta-cells and enhanced expression of proinflammatory cytokines. *In vitro* research indicates cytokine-induced ER stress can lead to increased exosome secretion by islet cells and subsequently increased exosomal proteins such as the chaperones calreticulin, ORP150 and Gp96, which can induce immune responses *via* enhanced phagocytosis and adjuvant capacity (45, 54, 55). The increased secretion of exosomes during ER stress may be explained by two theories. First, given their role in intercellular communication, the upregulation of exosomes may deliver ER stress conditions to neighboring cells. Second, exosomes may serve as vehicles for the disposal of unneeded cell material in response to ER stress to regain homeostasis. Another *in vitro* study indicated that exosomes containing miR-29b released from beta-cells can stimulate the secretion of IFN-α, IL-10, and IL-6 by splenocytes from NOD mice (48). Also, *in vitro* study indicates that the mouse insulinoma-derived microparticles also exert a strong adjuvant effect to induce the secretion of inflammatory cytokines, including IL-6 and TNF-α, *via* a MyD88-dependent pathway. *In vivo* experiments indicate that immunization with insulinoma-derived exosomes can cause insulitis in nonobese diabetes-resistant mouse models and that EXO-reactive Th1 cells and marginal zone-like B cells are detected in prediabetic NOD female mice (50, 51). Additionally, islet-derived mesenchymal stem cells (MSCs) can release highly immunostimulatory exosomes that can cause T cell-mediated destruction of the pancreatic islets in NOD mice (7). All these findings suggest that abnormal release of exosomes may trigger early inflammation and autoimmunity in the islets. However, whether these phenomena exist in human body and play a role in physiological process await further investigation.

On the other hand, exosomes derived from the immune system may lead to dysfunction and death of beta-cells (**Table 1**). A recent study indicated that exosomes containing specific miRNAs, including miR-142-3p/5p and miR-155, released by T cells can trigger apoptosis and chemokine gene expression in islet beta-cells of NOD mice (52). These chemokines, including Ccl2, Ccl7, and Cxcl10, are involved in the recruitment of immune cells and the promotion of beta-cell death in response to autoimmune attack. But because human T1DM has some distinct features compared to NOD mice, future studies need to clarify the adoptability in human body. Moreover, another study indicated that plasma-derived exosomes from patients with T1DM exhibit deregulated miRNAs and that these miRNAs are involved in the progression of T1DM (56). Subsequent functional analysis demonstrated that human islets coincubated with exosomes from T1DM patients showed decreased insulin output in the second phase in response to glucose stimulation. This finding suggests that exosomes and their content may serve as a new communication mediator between the immune system and insulin-producing beta-cells.

In addition, exosomes can deliver biological information between pancreatic beta-cells. One study indicated that the miRNA content of exosomes originating from beta-cells is regulated by inflammatory mediators, and incubation with

these cytokine-induced exosomes leads to naïve beta-cell apoptosis (46). This finding provides a novel potential communication mode to coordinate the activity of beta-cells in addition to direct cell-to-cell contact and the release of signaling molecules with autocrine and paracrine functions (57, 58). Moreover, another study indicated that low-dose cytokines can stimulate the secretion of exosomes carrying neutral ceramidase (NCDase) from INS cells and that these NCDase-containing exosomes can inhibit apoptosis induced by proinflammatory cytokines at a high concentration. Similarly, NCDase packaged in exosomes secreted from beta-cells can ameliorate palmitate-induced apoptosis in INS-1 cells (59). Although this discovery is more strongly associated with T2DM, it also suggests that horizontal message transfer between beta-cells *via* exosomes does exist and may play an important role in the pathological process of the pancreatic islets. However, all these findings are drawn based on cell experiments, *in vivo* studies are required to elucidate whether this mechanism also exists in physiological conditions and evaluate the relative contribution in mediating lateral communication between beta cells.

Notably, given that almost all cell types can secrete exosomes, there must be other pathogenic mechanisms apart from the communication mode mentioned above in the development of T1DM. For example, studies have shown that compared with those from healthy controls, breast milk-derived exosomes from mothers with T1DM contain different levels of miRNAs, and pathway analysis indicates that these miRNAs are involved in the modulation of the infant immune system (60). However, whether this increases the risk of T1DM in infants is unknown.

In conclusion, based on current knowledge, exosomes may play a critical role in the onset and development of T1DM by delivering biological information, at least between beta-cells as well as between the pancreatic islets and the immune system. Previous studies have demonstrated that a complex network formed by exosomes may collectively contribute to the onset of T1DM, but how much of a role exosomes can play remains further research.

Besides T1DM, exosomes also play a role in other autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and Sjogren's syndrome (61). It is not surprising because exosomes can be secreted by almost all cell types and has multiple biological functions, such as intercellular as well as interorgan communication and modulation of immune responses. In fact, exosomes may have broader effects on regulating physiological and pathological processes due to their universality and versatility.

EXOSOMES AS NOVEL BIOMARKERS OF T1DM

Before the manifestation of clinical symptoms, the underlying autoimmune changes of T1DM occur, and this symptomless period offers a great opportunity to predict and prevent disease progression (62). However, suitable biomarkers to identify and stratify the high-risk population and to evaluate the efficacy of intervention measures have not been developed, as the existing biomarkers often mark the late stage of T1DM when almost 90% of beta-cells have been lost. Currently, the combination of susceptible genes and islet autoantibodies is the most useful biomarker to predict T1DM risk (63). Previous studies have identified more than 50 candidate loci; a minority of genes (*HLA*) have large effects, but a majority of these genes have small effects (2). HLA genes confer the greatest risk for the development of T1DM, and the *HLA-DR* (DR3/4) and *HLA-DQ* (DQ8) genotypes are mostly used to predict the risk of developing islet autoimmunity (64, 65). Furthermore, combined evaluations of other risk genes with smaller effect sizes than *HLA* do remarkably improve sensitivity and specificity for the identification of high-risk individuals. However, a study indicated that 90% of individuals identified through genetic markers never displayed autoimmunity, and less than 50% of cases were identified by a combination of genetic markers (66).

The appearance of autoantibodies usually precedes the clinical manifestation of T1DM by months to years (67). The major circulating autoantibodies against beta-cell peptides and proteins include GAD65, IA-2, insulin, and zinc transporter 8 (ZnT8) (68–71). Although there is no evidence that these autoantibodies contribute to the pathogenesis of T1DM directly, it has been accepted that they are hallmarks of T1DM (65). At present, autoantibodies are used as biomarkers of T1DM in the clinic, and positivity for multiple autoantibodies is associated with a higher risk of T1DM regardless of family history (63). In fact, children positive for two or more autoantibodies almost inevitably develop diabetes. However, there are some limitations regarding the clinical application of these markers (67). For example, given that the time from seroconversion to diagnosis can span from weeks to decades, other biomarkers are needed before and after seroconversion. In addition, some patients never display these autoantibodies at diagnosis, and a subset of autoantibody-positive individuals will not develop clinical diabetes (72).

Currently, exosomes are viewed as potential biomarkers for diagnosing disorders such as tumors because the molecular cargo of exosomes can reflect the cell type and status of their releasing cells (15, 73). Furthermore, exosomes have additional advantages compared with traditional diagnostic methods, including (1) secretion in easily accessible biological fluids, such as urine and blood; (2) the ability to be preserved for a relatively long time at −80°C due to their stability; and (3) enhanced molecular stability in protease- and nuclease-controlled environments (43).

In the context of T1DM, previous studies have indicated that pathophysiological conditions in the pancreatic islets affect the composition of exosomes originating from beta-cells (**Table 2**) (46, 49). Profiling of exosomal RNAs derived from human islets with T1DM has demonstrated that RNAs are differentially expressed in cells subjected to treatment with proinflammatory cytokines compared to those without cytokine treatment, and these differentially expressed RNAs are associated with insulin secretion, necrosis, apoptosis, and calcium signalling (74). This study applying *ex vivo* stress model not only provides a comprehensive map of exosomal RNA from human pancreatic

TABLE 2 | Summary of findings on exosomes as biomarkers of T1DM.

Experimental subjects	Findings	References
Humans	Exosomal miRNAs may serve as potential circulating biomarkers of T1DM	(74)
Humans	Analysis of plasma-derived exosome miRNAs as novel diagnostic tools for T1DM	(56)
Humans	Circulating transplant islet-specific exosomes may be a novel diagnostic tool for recurrent autoimmune T1DM after islet transplantation	(75)
Humans and mice	Transplanted islet-derived exosomal miRNAs as biomarkers for monitoring immune rejection	(76)
Humans	Urinary excretion of AQP2 and AQP5 *via* exosomes as biomarkers for T1DM nephropathy	(77)
Humans	Urinary podocyte EVs may serve as early biomarkers of glomerular injury in T1DM	(78)
Humans	High levels of exosomal cytokines and angiogenic factors in plasma may serve as biomarkers of diabetic ocular complications	(79)
Humans	Increased cystatin B and altered protease profiles in urinary EVs may serve as biomarkers of kidney damage in T1DM	(80)
Rats	Decreased urinary exosomal regucalcin may serve as a biomarker of diabetic kidney disease	(81)
Humans	Urinary exosomal miR-145 may serve as a biomarker of T1DM with diabetic nephropathy	(82)
Humans	The WT1 protein in urinary exosomes may be an early noninvasive marker of diabetic nephropathy in T1DM	(83)

islets, but also can pin down the source of these circulating molecules. Among all the components, exosomal miRNAs are particularly attractive for developing novel biomarkers of T1DM (84). Global profiling analysis applying beta-cell lines and pancreatic islets has revealed that a subset of miRNAs is preferentially secreted in exosomes, while others are prone to be retained in cells (46, 85). Moreover, an *in vitro* study indicated that the miR-21-5p cargo inside EVs is increased in response to inflammatory cytokines and has promise as a future biomarker of T1DM (86). Subsequent research indicates that miR-21-5p from serum is increased in children with new-onset T1DM compared with healthy children, and interestingly, the total serum miR-21-5p is decreased among diabetic individuals, which proves the cargo within EVs is packed selectively (86). Future study should focus on identifying EV specific proteins that facilitate enrichment for EVs originated from beta-cell. With more practical significance, another study performed plasma-derived exosome characterization and reported a distinct miRNA signature in patients with long-duration T1DM, with seven differentially expressed miRNAs compared with healthy controls (56). However, the mean duration of diabetic participants in this study is 25.3 years, which weakens the potential diagnostic value of identified exosomal RNA. In the context of T1DM, exosomes have also been identified as biomarkers of diabetic complications, including nephropathy (78, 80, 82, 83) and retinopathy (87), and may be used for noninvasive monitoring of islet transplantation outcome (76).

EXOSOMES AS THERAPEUTIC TOOLS FOR T1DM

Nowadays, most patients with T1DM rely on life-long insulin administration, which can only relieve symptoms. According to existing knowledge about T1DM, the curable strategies lie in re-establishing immune tolerance, annihilate islet-reactive lymphocytes, and supplement the depleted beta-cells. Seeing that exosomes not only play a role in immune stimulation, but also in immune tolerance, they are emerging as an alternative tool to induce and rebuild auto-tolerance. Also, some stem cell-derived exosomes have been reported to protect beta-cell from autoimmune attack, slow disease progression, and improve the survival of transplanted islets.

The Advantages of Exosomes as Therapeutic Tool

Given that exosomes can exert biological effects on target cells, they are viewed as potential therapeutic agents (**Table 3**). Both *in vitro* and *in vivo* studies indicate that exosomes can transfer bioactive molecules between cells (101, 102). As a therapeutic delivery route for functional molecules, including RNA, DNA, and proteins, or synthetic drugs, exosomes can prevent cargo decomposition. For example, the clinical application of nucleic acids as drugs has been impeded because they are easily degraded. However, this problem can be solved by packaging RNAs and their mimics inside exosomes. A study indicated that two miRNAs, miR-106b-5p, and miR-222-3p, contribute to bone morrow transplantation (BMT)-induced beta-cell regeneration in mouse models of insulin-deficient diabetes, which may lead to the development of new therapeutic tools for diabetes (90). In addition to protecting the cargo from enzymatic degradation, the use of exosomes as therapeutic vectors has some other advantages, including (1) the ability to be isolated from patients themselves to avoid an immune rejection response; (2) a widespread distribution due to their liposolubility and ability to cross the intact blood-brain barrier; (3) the ability to be modified to target specific cell types by carrying special surface proteins or receptors; and (4) a relatively long half-life in the body (44, 103–105).

Stem Cell-Derived Exosomes and T1DM

Mesenchymal stem cells (MSCs), which can be also defined as multipotent stromal cells, possess self-renewal ability and can differentiate into other tissues. MSCs are capable to remodel the injured and inflammatory tissues and maintain homeostasis of microenvironment by directly differentiating into required cell types or secreting bioactive and soluble factors. In addition, some evidences indicate MSCs can suppress excessive immune response, such as activation of T cells and B cells, *via* their paracrine ability (106, 107). These immune regulatory

TABLE 3 | Summary of findings on exosomes as a potential therapeutic strategy for T1DM.

Experimental subjects	Findings	References
Human MSCs and PBMCs	MSC-derived MVs inhibit inflammatory T cell responses in the islets *via* induction of regulatory dendritic cells in T1DM	(88)
STZ-induced mouse model of T1DM	Exosomes released by adipose tissue-derived MSCs exert immunomodulatory effects upon T cells and ameliorate clinical symptoms of T1DM	(89)
Human pancreatic islets	Islet-derived EVs are involved in beta cell-endothelium cross-talk and the neoangiogenesis process and may benefit engraftment of transplanted islets	(85)
Mouse model of insulin-deficient diabetes	Exosomal miR-106b and miR-222 derived from transplanted bone morrow promote beta-cell proliferation and ameliorate hyperglycemia	(90)
STZ-induced rat model of T1DM	Stem cell-derived exosomes may regenerate beta-cells through the Pdx-1 pathway	(91)
STZ-induced rat model of T1DM	Exosomes derived from MSCs exert therapeutic and regenerative effects upon the pancreatic islets	(92)
Rat model of diabetic nephropathy	Exosomes released by human urine-derived stem cells prevent kidney injury in rats with T1DM	(93)
STZ-induced rat model of T1DM	Adipose tissue-derived MSC exosomes improve erectile function in diabetic rats	(94)
Rat model of T1DM	Exosomal miR-145 released by bone morrow stromal cells exerts neurorestorative effects in diabetic rats with stroke	(95)
STZ-induced diabetic rat model	Exosomes released by human endothelial progenitor cells promote cutaneous wound healing in diabetes	(96)
NOD *scid* gamma mouse model	MSC-derived exosomes improve islet transplantation by enhancing islet function and inhibiting immune rejection	(97)
STZ-induced diabetic mouse model	Exosomes released by bone morrow MSCs improve diabetes-induced cognitive impairment	(98)
Transgenic mouse model	Hsp20-engineered exosomes may be a potential therapeutic agent for diabetic cardiomyopathy	(99)
C57BL/6J mouse model	Exosomal miRNA let7c derived from MSCs attenuates renal fibrosis in diabetes	(100)
Rat model of T1DM	Exosomes derived from human urine-derived stem cells prevent T1DM kidney complications	(93)

characteristic of MSCs has been studied for the treatment of autoimmune disorders, such as T1DM, multiple sclerosis and inflammatory bowel disease (108). Though MSC therapy has great therapeutic potential for multiple diseases, it still has several critical limitations, such as high cost, low reproducibility, and safety issues. Inspiringly, EVs, including exosomes, seem to mirror biophysical characteristics of parent cells and convey the cell functions. Some studies have indicated that the protective paracrine effects of MSCs are at least partially mediated by EVs, that is, EVs have homologous anti-inflammatory and regenerative effects as MSCs (109).

To date, accumulated research suggests that stem cell-derived exosomes possess congenital therapeutic potential and might protect pancreatic beta-cells from autoimmune assault, thus ameliorating disease progression (105, 110). It has been reported that exosomes isolated from menstrual blood-derived MSCs enhance beta-cell regeneration and insulin secretion through the pancreatic and duodenal homeobox 1 pathway in rat models of T1DM (91). However, there is no significant impact on non-fasting blood glucose observed, indicating the increased insulin might still be below the normal level. Therefore, further investigation focused on identifying administration dose and duration of therapy of exosomes may be necessary. Additionally, a recent study indicated that streptozotocin (STZ)-induced diabetic rats treated with exosomes derived from MSCs display lower blood glucose levels and higher plasma insulin levels, indicating the regeneration of insulin-producing beta-cells (92). Histopathological examination also proved that there is an increase in the size and number of beta-cells with decreasing fibrosis and inflammation of the islets. Moreover, in comparison with their parent cells, MSC-derived exosomes showed superior therapeutic and regenerative results (92). In fact, some researchers have stated that exosomes can be used as an alternative to whole stem cell therapies because they are safer, faster, and easier to inject, with more efficient outcomes and longer storage times than stem cells (92, 111). However, more research may be needed to elucidate the reason why exosomes encompass greater regenerative ability than MSCs themselves, and which substances inside exosomes actually function. In addition to enhancing beta-cell regeneration and function, MSC-derived exosomes also have immunomodulatory effects (112–114). *In vitro* studies demonstrate that EVs derived from bone marrow MSCs induce regulatory dendritic cells and inhibit the proinflammatory responses of T cells against the GAD antigen in patients with T1DM (88, 115). *In vivo* experiments indicated that exosomes derived from adipose tissue-derived MSCs exert protective effects on STZ-induced T1DM mice by increasing the population of regulatory T cells and their products without increasing the proliferation index of lymphocytes (89). All these findings suggest that EVs can mimic the immunoregulatory properties of MSCs and better understanding of involved mechanisms will benefit cell-free therapeutic application.

Stem Cell-Derived Exosomes and T1DM Complications

Moreover, some animal experiments have indicated that exosomes can also ameliorate diabetic complications (99, 116). Rat bone marrow MSC-derived exosomes can improve cognitive impairment in STZ-induced diabetic mice by repairing damaged neurons and astrocytes, thus reversing dysfunction (98). Although this study shows the exosomes released from MSCs boost impaired neuronal functions, the involved specific proteins or RNA are not identified. Another study showed that exosomal miR-let7c derived from MSCs attenuated kidney injury by preventing renal fibrosis in C57BL/6J mice, which are susceptible to diet-induced obesity and T2DM, with unilateral ureteral obstruction (100). An *in vivo* study indicated that exosomes released by human urine-derived stem cells can prevent podocyte apoptosis and promote cell survival as well

as vascular regeneration in rats with T1DM (93). Future studies to clarify the underlying mechanisms and pathways of exosomes on preventing diabetic kidney impairment are necessary. Additionally, exosomes isolated from human endothelial progenitor cells can facilitate cutaneous wound healing by promoting angiogenic activity and vascular endothelial function in diabetic rats and mice (96, 117). Similarly, future research should focus on determining the exact components within exosomes contributing to wound healing of diabetic patients before clinical use. In addition to biomolecule delivery, exosomes can also be applied to deliver synthetic drugs, such as curcumin, that can ameliorate neurovascular dysfunction after stroke in T1DM (43). In conclusion, the animal studies mentioned above indicate that stem cell-derived exosomes have great potential to treat T1DM and diabetic complications and further investigation should elucidate their clinical value in patients with T1DM. However, it should be considered that exosomes could still allow existing tumors to invade the immune system because they can promote cell survival, stimulate angiogenesis, and modulate immunity, although they exhibit a greatly decreased risk of carcinogenesis and maldifferentiation compared with MSCs (118, 119).

Exosomes and Islets Transplantation

Encouragingly, some research suggests that exosomes might promote the survival of transplanted pancreatic islets and enhance the efficiency of this treatment (120). The cross-talk between endothelial cells and beta-cells is critical for islet transplantation because it is associated with the revascularization process. *In vitro* experiments indicate that human islet-derived exosomes carrying angio-miRNAs can be captured by intraislet endothelial cells and favor angiogenesis and engraftment (85). Further studies should focus on evaluating whether they can be applied in inhibiting ischemia-reperfusion injury in solid organ and cell transplantation. It has also been reported that MVs released from endothelial progenitor cells can activate an angiogenic program and sustain vascularization in SCID (severe combined immunodeficient) mice, which lack both T and B lymphocytes (121). Furthermore, islet-derived exosomes have been observed to induce the expression of proangiogenic and antiapoptotic factors and inhibit antiangiogenic and proapoptotic molecules in islet endothelial cells (85). In addition to promoting revascularization, exosomes can improve islet transplantation through immunomodulatory effects. A study indicated that MSC-derived exosomes can improve islet transplantation by enhancing regulatory T cell function and inhibiting peripheral blood mononuclear cell (PBMC) proliferation (97). For safety concerns, the dose of factors inside exosomes needs to be accurately investigated. In summary, in the context of islet transplantation, exosomes may represent an exciting new therapy not only for the improvement of revascularization but also for the induction of transplant tolerance.

There are several practical problems that should be taken into consideration before any clinical use. First, the cell origin of exosomes affects their distribution, suggesting organotropic characteristics (104, 122). Therefore, modification of the exosome membrane may increase binding to specially targeted cells. Moreover, the route of administration, including intraperitoneal or intramuscular administration, can decrease the accumulation of exosomes in the liver, potentially leading to a higher concentration in target organs, such as the pancreas (122). Finally, the timing and clearance pattern of exosomes should be investigated. Studies show that macrophage-depleted mice display slower disappearance of injected exosomes, suggesting that macrophages may be associated with exosome clearance (123).

CHALLENGES AND PROSPECTS

In the past few decades, exosomes have shown great potential in development of autoimmune diseases, including T1DM. However, their basic and applied research is still in the early stage, and many challenges must be overcome. First, in all studies associated with exosomes, the isolation, purification and identification process is the first and the most important step. Nowadays, the most effective technique to get exosomes is differential ultracentrifugation, which cannot obtain exosomes with 100% purity. Exosomes in most T1DM studies actually represent mixed EV populations, mainly including exosomes and MVs. Therefore, the further research should focus on developing the specific markers to distinguish different subtypes of EVs. In addition, one issue in the application of exosomes for diagnostic markers is process portability, so unified methodologies for the isolation, purification, and characterization of exosomes should be generated before translation to clinical practice. Moreover, exosomes isolated from biofluids such as blood derive from multiple different tissues and organs. However, no clear surface markers have been identified for exosomes from different cell types. In the context of T1DM, the abnormality of both pancreatic islets and immune system contributes to its pathological process and many kinds of cells take part in its onset and development. Thus, developing approaches to determined origination of exosomes will be beneficial to clarify their functions in T1DM and reveal the underlying mechanisms of this disease. Finally, exosomes have shown "double-edged sword" characteristic, not only promoting, but also suppressing diseases progression, such as tumors (124). Therefore, identifying exosomes subgroups on the basis of their functions is equally critical.

CONCLUSION

Currently, the treatment of T1DM and its related complications is associated with an enormous economic burden for both society and individuals. Early identification of high-risk individuals do not catch T1DM is critical to implement timely preventive measures and avoid or delay disease exacerbation. Additionally, more comprehensive knowledge of the pathophysiological process will help us treat the root cause of diabetes rather than relieving its symptoms only. For the past few years, EVs, especially exosomes, have emerged as important agents mediating intercellular communication. Exosomes take

part in not only physiological processes in the body but also pathological conditions. Accumulated evidence has shown that they are involved in the onset and development of diabetes and that disease conditions alter the number and cargo of exosomes. Therefore, a better understanding of exosomes will help us reveal the underlying pathogenic mechanisms of T1DM, provide novel biomarkers for diagnosis, and lead to the development of new therapeutic strategies.

AUTHOR CONTRIBUTIONS

HP searched references, wrote the first draft of the paper, and revised the text. SL, YAX, YIX, XL, and GH critically revised the text and provided substantial scientific contributions. ZX and ZZ proposed the project and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Granulocytic Myeloid-Derived Suppressor Cell Exosomal Prostaglandin E2 Ameliorates Collagen-Induced Arthritis by Enhancing IL-10$^+$ B Cells

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The results of recent studies have shown that granulocytic-myeloid derived suppressor cells (G-MDSCs) can secrete exosomes that transport various biologically active molecules with regulatory effects on immune cells. However, their roles in autoimmune diseases such as rheumatoid arthritis remain to be further elucidated. In the present study, we investigated the influence of exosomes from G-MDSCs on the humoral immune response in murine collagen-induced arthritis (CIA). G-MDSCs exosomes-treated mice showed lower arthritis index values and decreased inflammatory cell infiltration. Treatment with G-MDSCs exosomes promoted splenic B cells to secrete IL-10 both *in vivo* and *in vitro*. In addition, a decrease in the proportion of plasma cells and follicular helper T cells was observed in drainage lymph nodes from G-MDSCs exosomes-treated mice. Moreover, lower serum levels of IgG were detected in G-MDSCs exosomes-treated mice, indicating an alteration of the humoral environment. Mechanistic studies showed that exosomal prostaglandin E2 (PGE2) produced by G-MDSCs upregulated the phosphorylation levels of GSK-3β and CREB, which play a key role in the production of IL-10$^+$ B cells. Taken together, our findings demonstrated that G-MDSC exosomal PGE2 attenuates CIA in mice by promoting the generation of IL-10$^+$ Breg cells.

Keywords: granulocytic myeloid-derived suppressor cells, exosomes, prostaglandin E2, collagen-induced arthritis, IL-10$^+$ Breg cells

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INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation in the synovium. Collagen-induced arthritis (CIA) is a well-established experimental model of human RA in mice, which exhibit severe swelling of the paws, synovial hyperplasia and joint ankylosis after type II collagen immunization (1–3). These phenomena are the result of the infiltration of lymphocytes into the synovium and the production of collagen-specific IgG autoantibodies by B

cells (4). Recently, a new subset of B cells termed regulatory B cells (Breg cells) were identified that can secrete interleukin-10 (IL-10) to inhibit the production of proinflammatory cytokines and restrain excessive immune responses (5, 6). The adoptive transfer of Breg cells has been reported to suppress the development of arthritis (7). Additionally, B cell-derived IL-10 is necessary for protecting NOD mice from Type 1 diabetes (T1D), as the infusion of NOD-IL-$10^{-/-}$ B cells has no effect on disease improvement (8). Therefore, the use of Breg cells may be an effective means of treating RA in the future.

Myeloid derived suppressor cells (MDSCs) are heterogeneous immature myeloid cells that possess a strong ability to suppress immune responses (9, 10). According to their morphological characteristics and the expression of Gr-1, murine MDSCs are divided into two major populations: granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs) (11, 12). Both cell types can expand and accumulate under pathological conditions, including tumor formation, inflammation and pathogen infection (13, 14). In addition these cells can express immuno-suppressive factors such as arginase-1 (Arg-1), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGF-β) and cyclooxygenase-2 (COX-2) (15). COX2 is an inducible enzyme that can be activated in variety of cells under specific conditions (16). As COX2 can convert arachidonic acid (AA) into prostaglandin E2 (PGE2), the level of PGE2 is typically used as an indicator of COX2 activity (17). PGE2 is a proinflammatory mediator produced by cancer and myeloid cells that acts on G-protein-coupled receptors (18, 19). MDSCs have been reported to express high levels of COX2 and are a major source of PGE2. This positive feedback loop between PGE2 and COX2 plays important role in the function and stability of MDSCs (20, 21). Studies have shown that PGE2 can regulate Th2-mediated cytokine spectrum, especially with respect to promoting IL-10 production (22, 23). PGE2 can also exert anti-inflammatory activities on macrophages and dendritic cells (24, 25). These effects are highly correlated with the inhibition of glycogen synthesis kinase 3 (GSK3) induced by PGE2. GSK3 is a ubiquitous serine/threonine kinase that has been shown to be a convergence point for many signaling pathways, with GSK3 being able to phosphorylate over 50 substrates to regulate cellular function (26). Furthermore, the effective use of GSK3 inhibitors to treat CIA has been reported due to their ability to alleviate joint swelling and eliminate histologically graded damage (27).

Exosomes are nanosized vesicles produced by multivesicular bodies (MVBs) that harbor multiple membrane proteins, such as CD9, CD81, and CD63 (28). Small molecules, such as water-soluble proteins, nucleic acids, and lipids can be transported from donor to recipient cells through exosomes, allowing for the exchange of substances and information between donor and recipient cells (29). Exosomes exhibit better stability than the parent cells in the treatment of diseases. Furthermore, studies have shown that the exosomes of immature DCs treated with immunoregulatory cytokines can inhibit inflammation and alleviate the course of CIA in the rat footpad model of delayed hypersensitivity (30). Therefore, exosomes may be used in potential therapies for arthritis and other autoimmune diseases in the future (31).

In the present study, we showed that G-MDSCs exosomes (G-exo) could attenuate the disease process of murine CIA. Furthermore, G-exo could promote IL-10^{+} Breg cell generation *in vivo* and *in vitro*, which was primarily associated with exosomal PGE2. Taken together, the results of the present study demonstrated that G-MDSC exosomes are a potentially novel mediator for the treatment of CIA mice.

Abbreviations: G-MDSCs, granulocytic-myeloid derived suppressor cells; RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, type II collagen; IL-10, interleukin-10; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; glycogen synthesis kinase-3β, GSK-3β; CREB, cAMP response element binding protein; Breg, regulatory B cells; Tfh, follicular helper T.

MATERIALS AND METHODS

Mice

DBA1/J mice (8–10 weeks old, male) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China), and C57BL/6 mice (6–8 weeks, male) were purchased from the Jiangsu University Animal Center (Zhengjiang, China). All animal experiments performed in the present study were approved by the Jiangsu University Animal Ethics and Experimentation Committee.

Induction and Assessment of Arthritis

Briefly, the DBA1/J mice were immunized by injecting 100 μg of emulsions that were acquired by bovine type II collagen (CII; Chondrex, WA, USA) emulsified with an equal volume of complete Freund's adjuvant (CFA, Sigma-Aldrich, St. Louis, MO, USA). On day 21, the mice received a secondary immunization with a booster emulsion prepared with CII and incomplete Freund's adjuvant near the primary injection site. To determine the effects of the G-exo treatment, mice received 100 μg of G-exo on days 18 and 24 after the first immunization. From day 21 on, mice were scored for signs of arthritis every 3 days. Each paw was evaluated and scored individually using a 0 to 4 scoring system as previously described (27).

Histopathologic Examination

Mice were sacrificed on day 42, and murine joint tissue specimens were obtained and fixed in 10% phosphate-buffered formalin for 3 days. Tissue sections (4-μm-thick) were stained with H&E to examine morphological features and perform histologic arthritis scoring.

Isolation of G-MDSCs

Tumor –bearing mice were established with the Lewis lung adenocarcinoma cell line. G-MDSCs were harvested from mouse spleens using G-MDSC isolation kits (Miltenyi Biotec, Cologne, DE) according to the manufacturer's protocol. The purity was evaluated by measuring the expression of Ly-6G and CD11b *via* flow cytometry (FCM).

Extraction and Identification of Exosomes

G-MDSCs were first cultured in plates (in R1640 medium supplemented with 10% fetal bovine serum ultracentrifuged at 100,000*g* for 16 h at 4°C) at 37°C under an atmosphere with 5%

CO_2. After 16 h, the culture supernatant was obtained. Subsequently, the cultures were centrifuged to remove debris as follows: 500 × g for 10 min at 4°C, 1,000*g* for 10 min and 10,000*g* for 30 min. Then, the supernatants were filtered through 0.22-μm pore filters (Millipore, Billerica, MA, USA). Exosomes were precipitated using an exosome extraction kit (System Biosciences, Palo Alto, CA, USA), dissolved in PBS and then stored at −80°C. During the purification of celecoxib-treated G-exo, the G-MDSCs used to extract exosomes were cultured in the presence of graded doses of celecoxib (Pfizer Inc., La Jolla, CA, USA). Subsequently, the purified G-exo were fixed and examined by transmission microscopy (Tecnai-12; Philips, Amsterdam, Netherlands) as previously described. The protein concentrations were determined by using a Micro BCA protein assay kit (Beijing ComWin Biotech, Beijing, China). In this study, we also prepared neutrophil-derived exo (Neu exo), which served as control for G-MDSC exo. The concentration and size distribution of the G-exo were measured *via* nanoparticle tracking analysis (NTA). The expression of the exosomal marker CD63, CD9, and the negative marker calnexin was measured by Western blot analysis.

Isolation and In Vitro Culturing of B Cells

For *in vitro* experiments, $CD19^+$ B cells were isolated from spleens using mouse CD19 microbeads (Miltenyi Biotec). Then, B cells were cultured in medium supplemented with 10 μg/ml LPS (Sigma-Aldrich) alone or together with G-exo.

Exosomes Labeling and Uptake

Exosomes were labeled with PKH67 (Sigma-Aldrich) according to manufacturer's instructions. Isolated B cells were first incubated with labeled exosomes for 6 h before being washed with exosome-depleted FBS and then pelleted by centrifugation for 5 min in 400 × g. The pelleted cells were then resuspended, after which red fluorescent dye PE (Bio-legend) was added to the cell suspension for 4 min. Subsequently, the reaction was stopped by the addition of an equivalent volume of exosome-depleted FBS, after which the cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, and then mounted with Hoechst nuclear stain.

Flow Cytometry Analysis

Single cell suspensions were immunostained with various combinations of fluorescent dye-conjugated antibodies against the following proteins: CD19, CD4, CXCR5, PD-1, CD138, and B220 (eBioscience, San Diego, CA, USA). For intracellular cytokine staining, single cell suspensions were simulated with 50 ng/ml of phorbol myristate acetate (Sigma-Aldrich), 1 μg/ml of ionomycin (Enzo, Farmingdale, NY, USA) and 2 μg/ml of monensin (Enzo) for 5 h. Then, the anti-IL-10 mAb (eBioscience) used to for intracellular staining of IL-10 according to the manufacturer's instructions, and the stained cells were analyzed by flow cytometry using a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA).

Quantitative Reverse Transcription PCR (RT-qPCR)

To assess gene expression in B cells, we first extracted total RNA from samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, cDNA was synthesized with a ReverTraAce qPCR RT kit (Toyobo, Osaka, Japan). The expression of IL-10 was assessed using the cDNA samples for RT-qPCR analysis based on SYBR green detection method (Bio-Rad, Hercules, USA).The sequences of the primers used are as follows: IL-10, 5-GGTTG CCAAGCCTTATCGGA-3 (forward), 5-ACCTGCTCCACTG CCTTGCT-3 (reverse).

ELISA

The levels of total IgG in murine serum samples and IL-10 in culture supernatant were measured using ELISA Ready SET-Go kits (eBioscience) following the manufacturer's protocol. The concentration of prostaglandin E2 (PGE2) was assessed using a Prostaglandin E2 ELISA Kit-Monoclonal (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions. This assay is based on the competition between PGE2-acetylcholinesterase conjugate for a limited amount of PGE2 monoclonal antibody. PGE2 was quantified using the equation obtained from the standard curve plot. Chick collagen type 2 was coated on the plate overnight at 4°C. The plates were washed with 0.05% Tween-20 in PBS and then blocked with 5% BSA for 1 h. Then the plates were washed for three times and added into diluted serum. The plates were incubated at 37°C for 1 h. Subquently, the plates were washed and added with HRP-labeled lgG antibodies. Finally, OPD peroxidase substrate was added into plates. The absorbance was measured at 450 nm.

Western Blot Analysis

Proteins extracted from cells were prepared as described previously. Then, the lysates were separated by 12% SDS-PAGE and transferred onto immobilon polyvinylidene difluoride (PVDF) membranes (Bio-Rad), which were then blocked with 5% BSA in Tris-buffered saline with Tween 20. The membranes were then incubated with specific rabbit antibodies against phosphorylated (p)-GSK-3β (Santa Cruz Biotechnology, Texas, USA), total (t)-GSK-3β (Wanleibio, Shenyang, China), phosphorylated (p)-cAMP response element binding protein (CREB) (Wanleibio, Shenyang, China) and total (t)-CREB (Wanleibio, Shenyang, China) followed by an incubation with the secondary HRP-conjugated goat anti-rabbit IgG (CST, Danvers, MA, USA) according to manufacturer's protocol. Finally, chemiluminescent detection reagent (Champion Chemical, CA, USA) was used to visualize the bands on the PVDF membranes.

Statistical Analysis

All data were analyzed with a two-tailed Student's t-test or one-way ANOVA using SPSS 16.0. When the p value was less than 0.05, differences were considered statistically significant.

RESULTS

Extraction and Identification of G-MDSCs Exosomes

G-MDSCs were isolated from the spleens and analyzed by flow cytometry. The results showed that the G-MDSC purity was greater than 95%, which met the needs for follow-up experiments (**Figure 1A**). Subsequently, G-exo were extracted

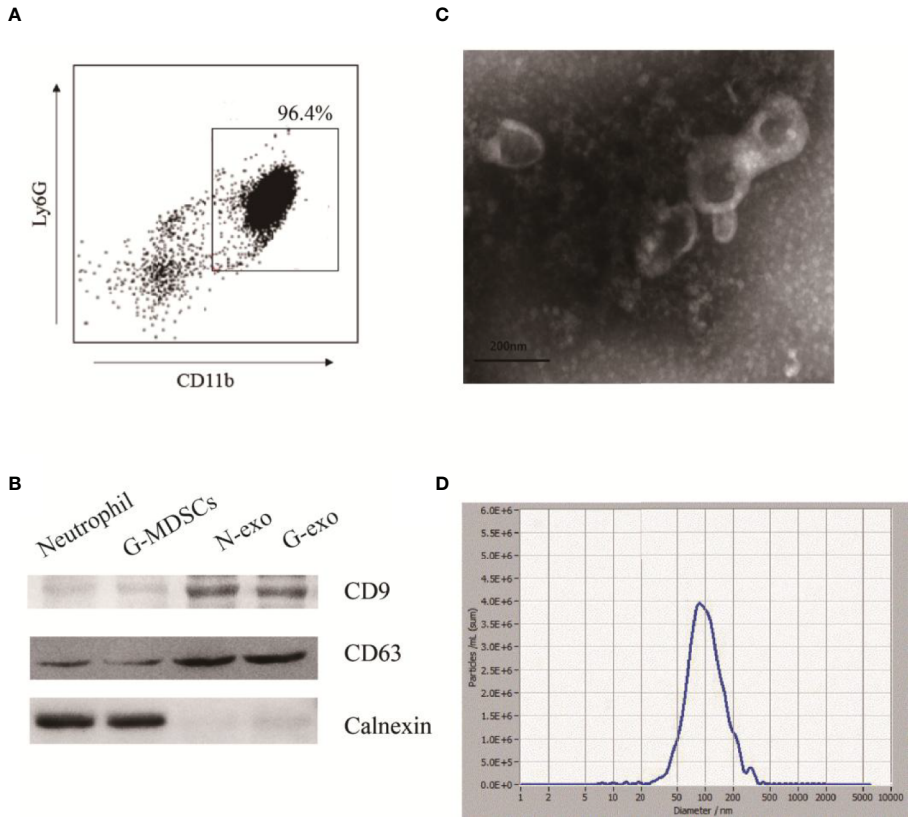

FIGURE 1 | Extraction and identification of G-exo. **(A)** G-MDSCs were sorted from mouse spleens using immunomagnetic beads. The expression of Ly-6G and CD11b was analyzed by flow cytometry. **(B)** The expression of CD63, CD9 and calnexin was assessed by Western blot analysis. **(C)** Representative transmission electron micrograph of G-exo (scale bar = 200 nm). **(D)** Particle size distribution of G-exo analyzed by nanoparticle tracking analysis (NTA). The presented data are from one of three independent experiments.

by differential centrifugation followed by the use of an exosome extraction kit according to the manufacturer's instructions. To identify the specific proteins expressed on exosomes, the extracted G-exo were evaluated by Western blot analysis. The results showed that CD63 was expressed in G-exo, whereas calnexin was not detected (**Figure 1B**). Further characterization by transmission electron microscopy and nanoparticle tracking analysis (NTA) indicated that the exosomes were saucer-like bilayer membrane vesicles with a particle size distribution peak of 99.6 nm (**Figures 1C, D**).

G-MDSCs Exosomes Attenuate the Development of CIA in Mice

To test whether G-exo can attenuate murine CIA, DBA1/J mice were administered an i.p. injection of G-exo (100 μg/mouse/injection) on days 18 and 24 after the first immunization. We observed that G-exo-treated mice were less susceptible to CIA than mice in the control group. First, the arthritis onset and disease progression were analyzed. As shown in **Figure 2B**, the mean arthritis index of G-exo-treated mice was lower than that observed in control mice. Although there was no significantly difference, CIA group mice had been completely suffered at 33 days, while mice treated with G-exo did not become fully ill until 39 days (**Figure 2C**). Second, the swelling caused by collagen stimulation in paws was strikingly reduced by G-exo treatment, while this change was not observed in the control group mice (**Figure 2D**). Third, histologic hind paw examinations revealed that substantial inflammatory cells infiltrated the articular cavity of control mice, which was accompanied by synovial cell proliferation and injury of the articular cartilage. In contrast, G-exo-treated arthritic mice showed significant improvement in the joint injury (**Figure 2E**). Furthermore, markedly lower levels of total IgG and anti CII antibodies were observed in the serum samples of G-exo-treated mice compared to that observed in the control mice (**Figures 2F, G**). Taken together, these results suggested that G-exo can attenuate collagen-induced arthritis, indicating their potential therapeutic effect.

G-MDSCs Exosomes Increase the Proportion of IL-10$^+$ B Cells but Decrease That of Plasma Cells and Follicular Helper T Cells in CIA

Considering the effect of G-exo on CIA mice described above, we next assessed whether G-exo treatment may have regulate the humoral immune response in arthritic mice. As shown in **Figure 3A**, the proportions of IL-10$^+$ B cells in the spleens and

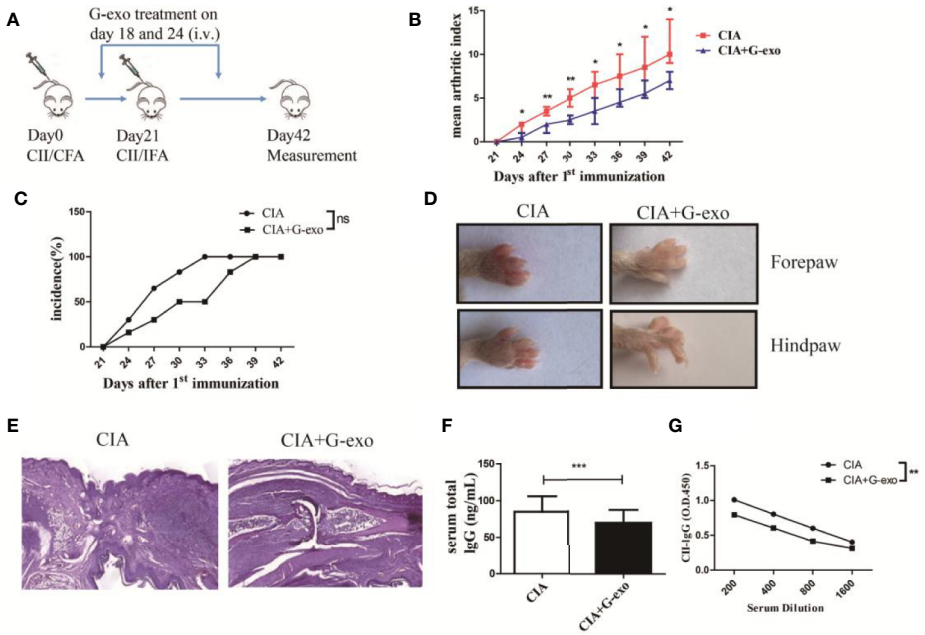

FIGURE 2 | G-exo attenuate collagen-induced arthritis in mice. **(A)** DBA1/J mice were immunized with CII/CFA on day 0 and boosted with CII/IFA on day 21. The treatment groups were intravenously injected with 100 μg of G-exo (CIA+G-exo) or phosphate buffered saline (control CIA) on days of 18 and 24. **(B)** The mean arthritis index was assessed once every three days after day 21 for the G-exo-treated or control mice according to the criteria for evaluation. **(C)** Incidence was assessed once every three days after day 21 for the G-exo-treated or control mice**. (D)** Images of hind paws and forepaws of the G-exo-treated or control mice. **(E)** Hind paws obtained from G-exo-treated or control mice were analyzed by H&E histologic examination. **(F)** Serum levels of total IgG were measured by ELISA. **(G)** Serum anti-CII antibody levels were measured by ELISA. Bar graphs show the means ± SD, *P < 0.05; **P < 0.01; ***P < 0.001; ns indicates no significance, (n = 6).

drainage lymph nodes (dLNs) of arthritic mice treated with G-exo were increased compared with that observed in control mice. The level of mRNA IL-10 was also upregulated in the spleens and dLNs in G-exo-treated CIA mice (**Figure 3B**). In addition, further results revealed that mice treated with G-exo had a decreased proportion of $B220^{-}CD138^{+}$ plasma cells in the dLNs, while no significant difference was observed in the spleens (**Figure 3C**). The proportion of $CD4^{+}CXCR5^{+}PD\text{-}1^{+}$ follicular helper T (Tfh) cells was decreased in the spleens and dLNs of arthritic mice treated with G-exo (**Figure 3D**). Thus, our data indicated that G-exo promoted $IL\text{-}10^{+}$ B cells generation but inhibited Tfh and plasma cells, which may contribute to the remission of arthritis.

G-MDSCs Exosomes Promote the Generation of IL-10–Producing B Cells In Vitro

Based on the regulatory effect of G-exo on arthritic mice, we examined the role of G-exo in B cells *in vitro*. Splenic B cells were first cocultured with PKH-67–tagged G-exo for 6 h. Subsequently, the membranes of cultured B cells were stained with PE dye. As shown in **Figure 4A**, the images revealed that G-exo could be taken up by B cells, as determined by confocal microscopy analysis. Next, B cells were cultured with LPS (10 μg/ml), G-exo (30 μg/ml), or with a combination of LPS and different concentrations of G-exo for 48 h. IL-10–producing B cells were analyzed by FCM, and the combination (32) of G-exo and LPS was observed to increase the proportion of IL-10–producing B cells *in vitro* (**Figure 4B**). These results further confirmed that the level of IL-10 mRNA expression was increased by the combination treatment of G-exo and LPS (**Figure 4C**). These findings confirmed that G-exo promoted the production of IL-10–producing B cells *in vitro*.

Exosomal Prostaglandin E2 Produced by G-MDSCs Is Key for the Production of IL-10–Producing B Cells

G-MDSCs were cultured in medium alone or with graded doses of celecoxib, a COX-2 inhibitor, for 16 h. First, the celecoxib was shown to have no effect on the number and viability of G-MDSCs (**Supplemental Figure 1**). The results showed that celecoxib could effectively reduce the expression of COX-2 in a dose-dependent manner (**Figures 5A, B**). As COX-2 is a biosynthetic enzyme involved in the synthesis of PGE2, the levels of PGE2 in the MDSC supernatant and exosomes in the celecoxib treatment group were significantly lower than that observed in control or dimethyl sulfoxide (DMSO) groups, indicating that celecoxib reduced the level of PGE2 in G-MDSC exosomes (**Figures 5C, D**). As shown in **Figure 5E**, G-exo treated with celecoxib could no longer promote IL-10 production by B cells in the presence of LPS. This result was further confirmed by the levels of IL-10 being downregulated in G-exo treated with celecoxib group (**Figure 5F**).

The GSK3 signaling pathway has been reported to regulate IL-10 expression, and PGE2 treatment leads to GSK-3β phosphorylation

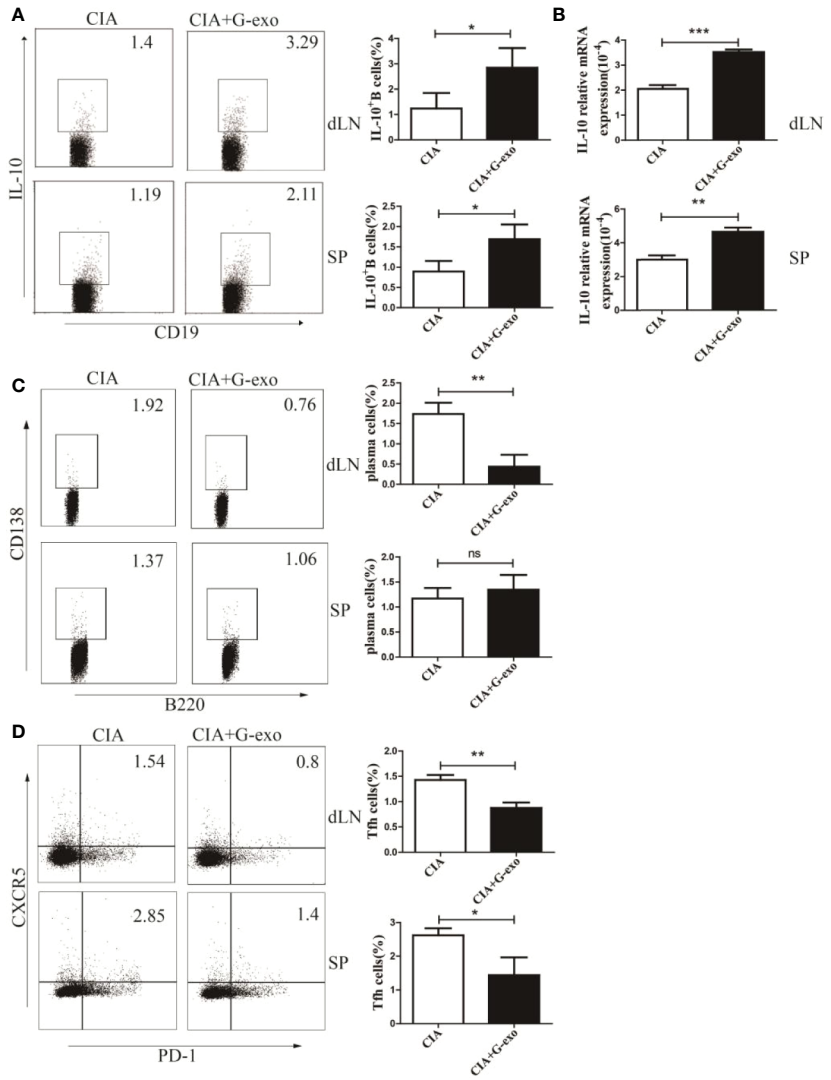

FIGURE 3 | G-exo increase IL-10$^+$ B cells but decreases the proportion of plasma cells and follicular helper T cells in CIA. **(A)** Proportions of interleukin-10 (IL-10)–producing B cells in drainage lymph nodes (dLNs) and spleens from CIA mice treated with G-exo (CIA+G-exo) or control mice (CIA) were analyzed by FCM. **(B)** The level of IL-10 mRNA in dLNs (up) and spleens (down) was determined by RT-qPCR. **(C)** The proportion of B220$^-$CD138$^+$ plasma cells in dLNs and spleens was analyzed by FCM. **(D)** The proportion of CD4$^+$CXCR5$^+$PD-1$^+$ follicular helper T cells in dLNs and spleens was analyzed by FCM. Bar graphs show the means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ns indicates no significance.

(28, 29). Therefore, we tested the activation of GSK-3β signaling molecules in B cells treated with G-exo or G-exo treated with celecoxib. We observed that G-exo upregulated GSK-3β phosphorylation at Ser9 and CREB at Ser133 in B cells, while GSK-3β and CREB phosphorylation was not significantly altered in G-exo treated with celecoxib (**Figures 5G–I**). These findings suggest that G-MDSC exosomal PGE2 may promote IL-10 production by B cells, which may involve the GSK-3β signal pathway (**Figure 6**).

DISCUSSION

In recent years, most studies established the prominent role of MDSCs in the regulation of immune responses during autoimmune pathology. Park et al. showed that adoptive transfer of MDSCs could decrease the number of Th1 and Th17 cells, both of which were widely considered as the important mediators in the pathogenesis of RA. However, they also found that transfer with MDSCs increased the number of Treg cells in spleen of CIA mice (32). Considering the types of MDSCs, sources of MDSCs and cell dose of MDSCs used in adoptive transfer, the exactly regulatory effect of MDSCs on various effector CD4$^+$ T cells populations in the CIA pathogenesis remains to be further researched. In addition, the effect of MDSCs on B cells is unclear. Exosomes are a type of membrane vesicle that can be transported to target cells, affecting the behavior and activity of the recipient cells. Zoller et al. have reported the immunomodulatory effect of MDSCs derived exosomes in mouse model of autoimmune

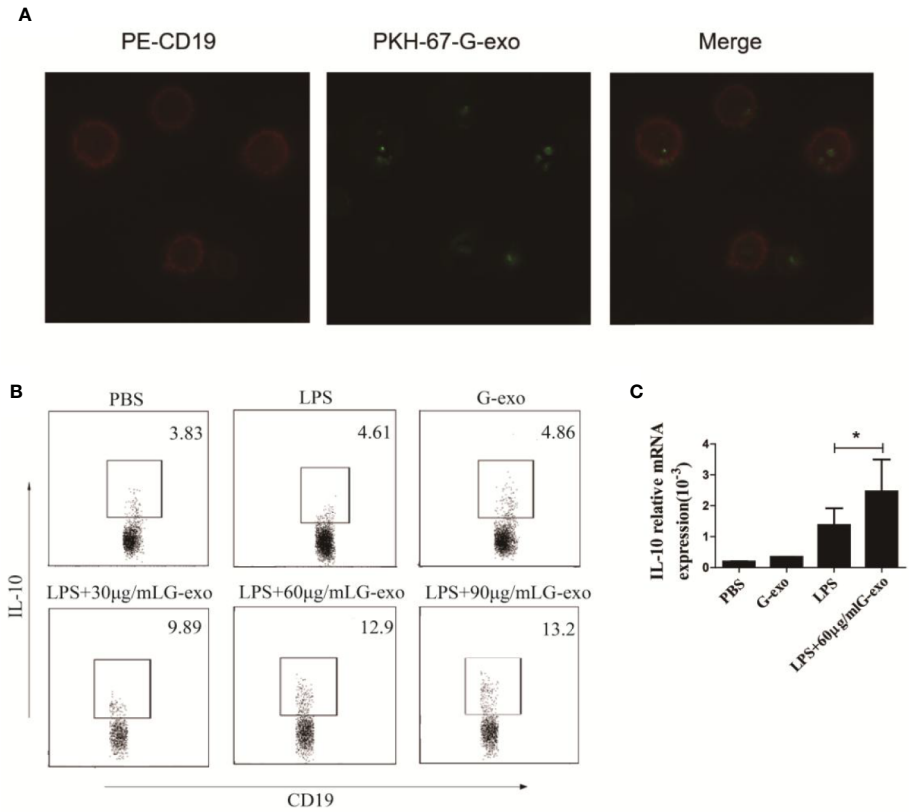

FIGURE 4 | G-exo promote the generation of IL-10$^+$ B cells *in vitro*. **(A)** Representative confocal microscopy images of B cells treated with PKH-67 (green)-tagged G-exo for 6 h. Cells were stained with the PE-anti CD19 mAb for the membrane. **(B)** Splenic B cells were cultured with LPS (10 μg/ml), G-exo (30 μg/ml), or with a combination of LPS and different concentrations of G-exo for 48 h. IL-10–producing B cells were analyzed by FCM. **(C)** Levels of IL-10 mRNA were determined by RT-qPCR. Bar graphs show the means ± SD. *P < 0.05. The presented data are from one of three independent experiments.

alopecia areata (33). Wang et al. also showed that G-exo could be used as a therapeutic approach because these exosomes could reduce DSS-induced-colitis (34). Our previous studies have already shown that the therapeutic effect of G-exo on CIA mice was by inhibiting the differentiation of Th1 and Th17 cells (35). Therefore, we further explored the role of G-exo on B cells in CIA mice. In the current study, the administration of G-exo indeed improved joint injury and slowed the disease process in CIA mice, and we noticed the decreased level of antibody in G-exo treated CIA mice. However, Crook et al. reported that PMN-MDSCs had no effect on B cell response but MO-MDSCs inhibited B cell proliferation and antibody production *via* iNOS and PGE2 in a contact-dependent manner (36). On the one hand, the reason for this discrepancy is due to the different sources of MDSCs, because G-MDSCs used in this experiment were isolated from tumor-bearing mice while MDSCs used in Crooks' experiment were isolated from CIA mice. On the other hand, it is attributed to different ways of action. In addition, we also discovered that G-exo could influence the frequency of plasma cells and Tfh cells. For the first time, we demonstrated that G-exo could reduce the proportion of Tfh cells in spleen and draining lymph nodes. Majority studies focused on the changes of Tfh cells in spleen of mice. However, MDSCs derived exosomes were more enriched in draining lymph nodes cells and were retained in skin-infiltrating leukocytes of AA mice for up to 48 h after tail vein injection (33). In view of the characteristic of MDSCs derived exosomes, it was essential to detect the effect of exosomes on lymph node cells in some autoimmune diseases. Over the past decade, B10 cells have been described as a functional subtype of immunosuppressive Bregs in the B cell population, characterized by the production of IL-10 in humans or mice. Park et al. reported MDSCs induced the expansion of regulatory B cells and ameliorated systemic lupus erythematosus severity in mice (37). Similarly, we also found G-exo could not only upregulate the proportion of IL-10–producing B cells in CIA mice but also induce IL-10$^+$ B cells *in vitro*. As previous studies reported, adoptive transfer of regulatory B cells into CIA models could ameliorate disease severity. Thus, our experiments provided a potential pathway of action for G-exo in the therapeutic treatment of CIA. However, the spleen was the predominant reservoir of B10 cells in mice. B10 cells and B10 precursor cells were rare within the lymph nodes and peripheral blood (38). Another guess had been put forward that immature B cells firstly differentiated into transitional B cells through Toll-like receptor, cytokines

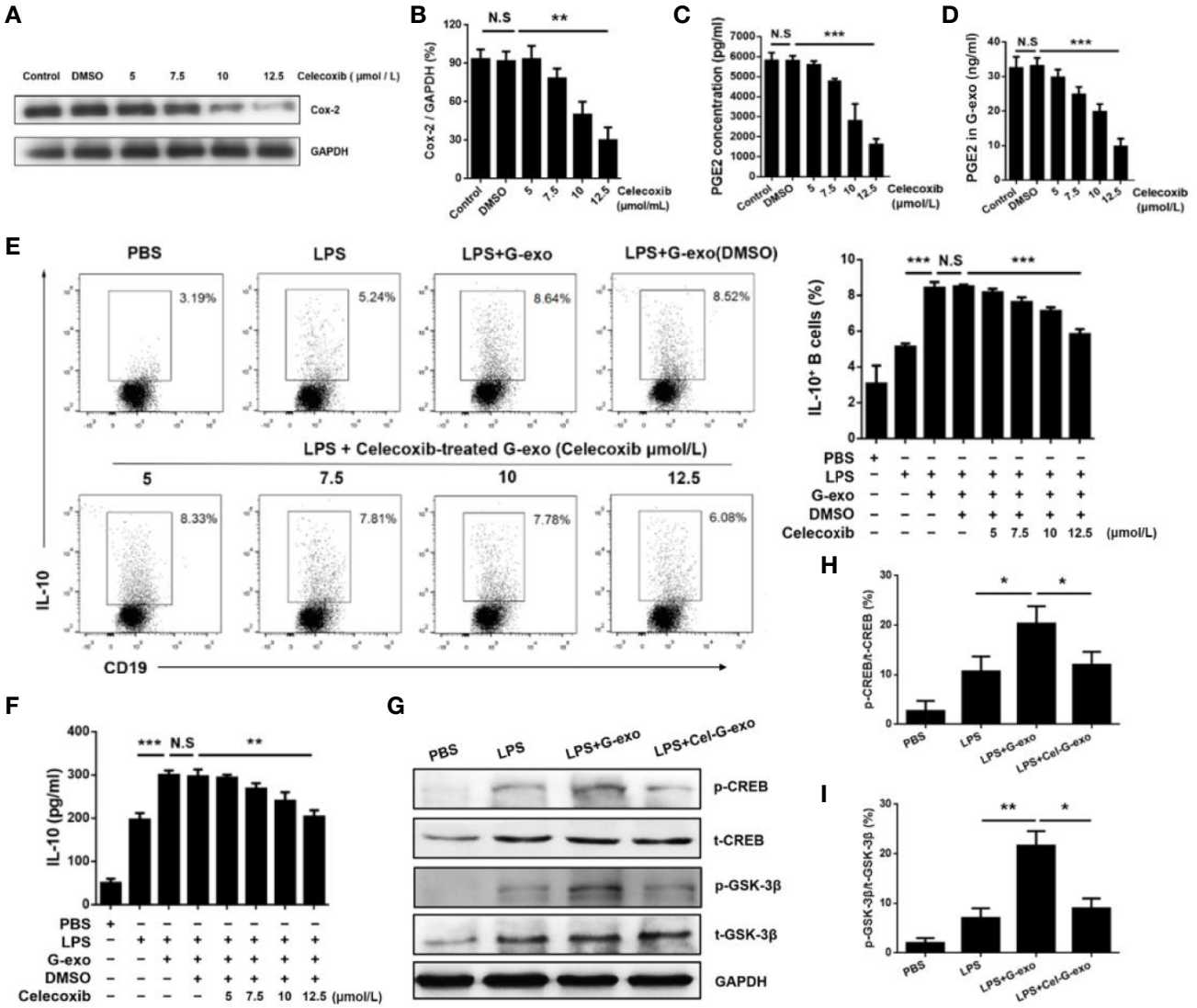

FIGURE 5 | G-MDSC exosomal PGE2 promotes IL-10$^+$ B cells**(A)** COX-2 expression in G-MDSCs was detected after treatment with the COX-2 inhibitor celecoxib. **(B)** The ratio of COX2 and GAPDH in each group was statistically analyzed. **(C)** The level of PGE2 in the G-MDSC culture supernatant treated with celecoxib was detected by ELISA. **(D)** The level of PGE2 in the G-exo treated with celecoxib was detected by ELISA. **(E)** IL-10–producing B cells after treatment with celecoxib-treated G-exo in the present of LPS were analyzed by flow cytometry. **(F)** The level of IL-10 in culture supernatant after treatment with celecoxib-treated G-exo was detected by ELISA. **(G)** The p-GSK-3β, T-GSK-3β, p-CREB, and T-CREB were detected by Western blot analysis. **(H)** The ratio of p-CREB and T-CREB in each group was statistically analyzed. **(I)** The ratio of p-GSK-3β and T-GSK-3β in each group was statistically analyzed. Bar graphs show the means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ns: indicates no significance.

stimulation and CD40 activations. Then, the transitional B cells differentiated into Breg cells (39). This suggests that Breg cell development is no longer limited to the spleen. Hence, the origin of B10 cells needs to be further researched.

Many previous studies have shown the biological effects of PGE2 and other cAMP-increasing substances on the cytokine profiles of macrophages and dendritic cells, which resulted in the suppression of inflammatory cytokines and enhanced levels of the anti-inflammatory cytokine IL-10 (40, 41). COX-2 is a key enzyme of PGE2 synthesis, and celecoxib is a selective COX-2 inhibitor. Therefore, celecoxib was chosen to inhibit the expression of PGE2 in G-MDSCs. We observed that G-exo contained a high concentration of PGE2, and treatment with celecoxib downregulated the level of PGE2 in G-exo. Interestingly, when PGE2 in G-exo was blocked by celecoxib, the ability of G-exo to induce IL-10$^+$ Breg cells was inhibited, suggesting that PGE2 is key factor for IL-10$^+$ Breg cell generation. Majority studies implied that the levels of PGE2 were higher in serum and synovial of arthritis patients and mice. However, Frolov et al. reported that PGE2 acted as important negative regulators in collagen antibody induced arthritis(CAIA) (42). Another study also showed that PGD2 prevent CIA from joint inflammation and destruction (43). Thus,

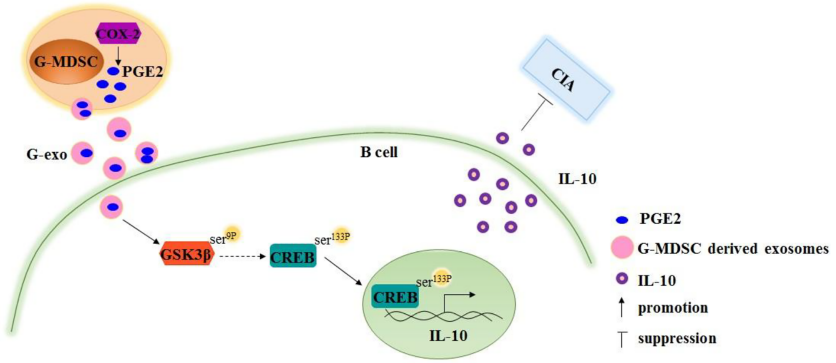

FIGURE 6 | Schematic image demonstrating that Granulocytic myeloid-derived suppressor cell exosomal prostaglandin E2 ameliorates collagen-induced arthritis by inducing IL-10^{+} B cells production *via* affecting GSK-3β and CREB phosphorylation.

our experiment aimed to imply that despite PGE2 were enriched in articular tissue or serum during the development of CIA, they could also play anti-inflammatory role in this process.

It has also been reported that PGE2 together with TLR agonists can promote the production of IL-10 in macrophages by activating the CREB-regulated transcriptional coactivator (44). Furthermore, in the PI3K/AKT pathway, AKT attenuates the activity of GSK3 by phosphorylation, which regulates the function of CREB (45). To elucidate a possible molecular mechanism by which G-MDSC exosomal PGE2 promotes Breg generation, we examined the levels of the signaling molecules GSK-3β and CREB. In the present study, we observed that G-exo enhanced the phosphorylation of GSK-3β at Ser9 and CREB at Ser133 in B cells, and the phosphorylation decreased when G-MDSC exosomal PEG2 was blocked by celecoxib. This finding suggests that the role of G-exo on Breg cells is mediated by the GSK-3β pathway. However, we did not observe the phenotype of IL-10–producing B cells in our studies, which are consistently characterized by the unique phenotype CD1d^{hi}CD5^{+} (46). In this study, 100 μg G-exo was injected into mice *via* the tail vein, which was consistent with most studies. It is still to be further explored if the therapeutic effect will increase with the amount of G-exo. However, Smyth et al. found that Balb/C mice were injected intravenously with 400 μg 4T1 cell-derived exosomes, and the mice rapidly developed dyspnea (47). Thus, the application of exosomes in clinical treatment is still limited by the dose, mode and toxicity of exosomes (48). But as shown, we believe it could still better complement the therapeutic effect of G-exo on CIA. Moreover, previous studies have shown that exosomes could be taken up by B cells, while PGE2 played a role by recognizing EP receptors on target cells, raising the question of whether PGE2 regulates the expression of PI3K by direct or indirect means.

In summary, we identified G-MDSCs derived exosomes as a potential mediator in the treatment of CIA mice. G-MDSCs derived exosomes mediate high levels of PGE2, subsequently promoting the generation of Breg cells with immunosuppressive function. These data further improve our understanding of the application of G-MDSCs derived exosomes in autoimmune arthritis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Jiangsu University Animal Ethics and Experimentation Committee.

AUTHOR CONTRIBUTIONS

XW, DZ, and HG performed experiments. JT, XT, and JM carried out the data analysis. SW, JT, and HX designed the experiments. XW and SW wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.588500/full#supplementary-material

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Olfactory Ecto-Mesenchymal Stem Cell-Derived Exosomes Ameliorate Experimental Colitis *via* Modulating Th1/Th17 and Treg Cell Responses

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Olfactory ecto-mesenchymal stem cells (OE-MSCs) are a novel population of resident stem cells in the olfactory lamina propria with strong immunosuppressive function. Exosomes released by MSCs are considered to carry various mRNAs, microRNAs and proteins from cells and function as an extension of MSCs. However, it remains unclear whether exosomes derived from OE-MSCs (OE-MSCs-Exos) possess any immunoregulatory functions. In this study, we found that OE-MSCs-Exos possessed strong suppressive function in $CD4^+$T cell proliferation, accompanied by reduced IL-17, IFN-γ and enhanced TGF-β, IL-10 secreted by T cells. In experimental colitis mice, treatment of OE-MSCs-Exos markedly alleviated the severity of disease, and Th1/Th17 subpopulations were remarkably reduced whereas Treg cells were increased after OE-MSCs-Exos treatment. Mechanistically, OE-MSCs-Exos were demonstrated to inhibit the differentiation of Th1 and Th17 cells, but promote the induction of Treg cells *in vitro*. Taken together, our findings identified a novel function of OE-MSCs-Exos in regulating T-cell responses, indicating that OE-MSCs-Exos may represent a new cell-free therapy for the treatment of IBD and other inflammatory diseases.

Keywords: exosome, inflammatory bowel disease, immunoregulation, T cells, olfactory ecto-mesenchymal stem cells

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, relapsing, and remitting inflammatory disorder of intestinal tract, including Crohn's disease and ulcerative colitis. The incidence of IBD has increased worldwide and challenges the public health (1). It has been acknowledged that the long-term chronic inflammation in the intestine can cause abscesses, fistulas, extraintestinal manifestations, and even colitis-associated cancer (2). Currently, the etiology of IBD is associated with the genetics, gut microbiota, immune responses, and the environmental factors (3). The aberrant immune responses are demonstrated to be closely associated with the chronic inflammation in IBD.

Accumulated studies have implied multiple adaptive immune cells are involved in the pathogenesis of IBD, including Th1, Th17 and regulatory T cells (Tregs) (4). To date, those traditional drugs, such as antibiotics, corticosteroids, and immunosuppressive agents, can only offer temporary remission but may result in the several side effects, including psoriasis, hypersensitivity, and drug-induced cytotoxicity (2). Thereafter, exploring novel alternative therapies for IBD is necessary.

Mesenchymal stem cells (MSCs) is a population of stromal cells with the capacity of self-renewal and multipotent differentiation potential. The immunomodulatory and anti-inflammatory properties of MSCs have provided powerful competition for MSCs in the cellular-based therapy for a variety of immune-mediated disorders, including autoimmune diseases and inflammatory diseases (5). In recent years, MSC-based therapeutic intervention has emerged as a promising strategy for the treatment of IBD in clinic trails (6, 7). However, the transplantation of MSCs has some shortcomings, such as the unstable phenotype from the different batches, cellular rejection, high costs in storage, transport, and recovery. Additionally, the transplanted MSCs can be modified *in vivo* by the complicated environment, leading to the invalid or unwanted effect in patients (8). Considering these drawbacks in the cell-based therapy, a novel cell-free therapy, MSC-secreted extracellular vesicles (EVs), including microvesicles and exosomes, have recently emerged as a powerful tool in the treatment of various diseases, such as degenerative diseases, graft-versus-host diseases, and inflammatory diseases (9–12). Exosomes are nano-sized EVs (40–100 nm in diameter) that possess remarkable physiological properties and originate *via* the inward budding of the membrane of late endosomes (13). Current researches indicate that exosomes derived from MSCs (MSC-Exos) mediate cell-cell micro-communication and transport paracrine factors during tissue repair and immune regulation, thus efficiently enhancing the therapeutic potency of MSCs in several diseases (14–18).

Olfactory ecto-mesenchymal stem cells (OE-MSCs) is a new type of stem cells resident in the olfactory lamina propria. As a population of stem cells, OE-MSCs possess the capability of self-renewal, high proliferation rate and multiple lineage differentiation potential (19, 20). Our previous data have identified that OE-MSCs exhibited powerful immunomodulation capacity and ameliorated the severity of murine collagen-induced arthritis *via* regulating T cell responses (21). However, our exploration further showed that the immunosuppressive function of OE-MSCs could be down-regulated by IL-17, indicating the endogenous inflammatory microenvironment could modify the transplanted cells and impair the therapeutic potential of OE-MSCs in clinical application (22). Therefore, as a novel cell-free therapy, OE-MSCs-derived exosomes (OE-MSCs-Exos) are supposed to provide multiple advantages over cell-based treatment, and might possess great therapeutic potential in inflammatory diseases.

In this study, we investigated the immunomodulatory function of OE-MSCs-Exos in regulating T-cell responses. We found that OE-MSCs-Exos exerted their immunosuppressive capacity *via* inhibiting Th1 and Th17 cell differentiation and promoting regulatory T (Treg) cell induction. By using the experimental colitis mouse model, OE-MSCs-Exos were demonstrated to alleviate the disease severity, accompanied by decreased Th1/Th17 cell responses and increased Treg cells *in vivo*. Thus, our findings identified the capacity of OE-MSCs-Exos in regulating T-cell functions, indicating that OE-MSCs-Exos may present an effective therapeutic tool for the treatment of IBD.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice at 6–8-week old were purchased from Experimental Animal Center of Yangzhou University. Mice were housed in a specific pathogen-free animal facility, and all the experiments were approved by the Committee on the Use of Live Animals in Research and Teaching of Jiangsu University.

Isolation and Culture of OE-MSCs

OE-MSCs were isolated from the nasal cavity of C57BL/6 mice as we previously described (21). Briefly, the olfactory epithelium tissue was cut into small pieces and cultured in flasks with the medium DMEM/F-12 supplemented with 15% fetal calf serum) (Gibco) for 7 days. After removing the non-adherent cells, the remaining cells were trypsinized and further expanded for 3 passages.

Osteogenic and Adipogenic Differentiation Assay

Osteogenic and adipogenic differentiation of OE-MSCs was analyzed as we previously described (21). For osteogenic induction, OE-MSCs were cultured in osteogenic induction medium for 3 weeks, and then the osteogenic differentiation was detected by alizarin red (Cyagen) staining. For adipogenic differentiation, OE-MSCs were cultured in adipogenic differentiation medium (Cyagen) for 14 days, and then cells were stained with oil red O (Cyagen).

Isolation of OE-MSCs-Exos

OE-MSCs were cultured in the medium with exosomes-depleted FBS for 48 h, and the supernatants were collected to extract exosomes by ultracentrifuged method. Exosomes were purified from the supernatants by differential centrifugation to remove cells and debris, and then the supernatants were ultracentrifuged at 100,000 g for 60 min at 4°C. After removing the supernatants, the exosomal pellets were washed in PBS and centrifuged at 100,000 g for another 60 min at 4°C, and then resuspended in PBS and stored at -80°C. The protein concentration of OE-MSCs-Exos was quantified using a micro-BCA protein assay kit.

Electron Microscopy

The transmission electron microscopy (TEM) analysis was performed as we previously described (23). Briefly, OE-MSCs-Exos suspension were fixed in 4% paraformaldehyde at 4°C for 60 min. Then, the exosomal pellets were applied to a formvar-

coated grid and negatively stained with 3% aqueous phosphotungstic acid. The TEM sample imaging was performed using transmission electron microscopy (Tecnai-12, Philips).

For scanning electron microscopy (SEM), exosomes were fixed with 3.7% glutaraldehyde and then dehydrated with ethanol. Sections were left to dry at room temperature for 24 h and then analyzed by SEM.

Nanoparticle Tracking Analysis

The sizes of OE-MSCs-Exos were measured by NTA using a ZetaView PMX 110 (Particle Metrix) and analyzed by the software ZetaView 8.04.02.

Western Blotting Analysis

Proteins extracted from the exosomes were separated by 12% SDS-PAGE, then transferred onto Immobilon polyvinylidene membranes (Bio-Rad) and probed with antibodies against CD9, CD63 and calnexin (Abcam) followed by chemiluminescent detection (Champion Chemical).

Flow Cytometric Analysis

For surface markers, MSCs were collected to stain with relevant fluorochrome-conjugated monoclonal antibodies (mAbs): anti-mouse CD29, CD90, CD44, CD34, CD45, and CD11b from eBioscience. Lymph nodes were isolated from mice and ground in culture medium. The suspensions were filtered through 70-μm cell strainers. For intracellular cytokine staining, single cell suspensions were stimulated with PMA (Sigma-Aldrich, 50 ng/ml), ionomycin (Enzo, 1 μg/ml), and monensin (Enzo, 2 μg/ml). After 5 h, cells were stained with anti-CD4 mAb (eBioscience), fixed, permeabilized, and stained with anti-IFN-γ or IL-17 mAbs (eBioscience) according to the Intracellular Staining Kit (Invitrogen, Carlsbad, CA) instructions. For Treg cells staining, anti-CD4, anti-CD25, and anti-Foxp3 mAbs (eBioscience) were performed following Foxp3 Staining Buffer Set (eBioscience) protocols. Flow cytometry was performed using the BD FACSCanto II (Becton Dickinson) and data were analyzed using FlowJo software (Becton Dickinson) (24).

T Cell Suppression Assay

Mouse $CD4^+$ T cells were sorted from wild-type mice using $CD4^+$T cell microbeads (Miltenyi Biotec), and the purity of $CD4^+$T cells was >95%. The isolated $CD4^+$ T cells labeled with carboxyfluorescein succinimidyl ester (CFSE, 5 mM; Invitrogen) were treated with or without OE-MSCs-Exos in the presence of anti-CD3 and anti-CD28 mAbs (eBioscience) for 72 h. CFSE fluorescence intensity was analyzed to determine the proliferation of $CD4^+$ T cells by flow cytometry.

Enzyme-Linked Immunosorbent Assay

Concentrations IFN-γ, IL-17, TGF-β, and IL-10 in the supernatants were detected by sandwich ELISA (eBioscience). All Assays were performed according to the manufacturer's instructions. Briefly, samples were incubated in 96-well plates pre-coated with the capture antibody for 2 h at room temperature, followed by incubation of biotin-conjugated detection antibody for 1 h. After washing, HRP Streptavidin was added and incubated for 30 min. Then, plates were washed and the TMB substrate was added. After 30 min, stop solution was added and absorbance was measured at 450 nm using a microplate reader (Biotek Winooski).

DSS-Induced Experimental Colitis

Male C57BL/6 mice were subjected to 2.5% DSS treatment from day 0 to day 4, followed by water treatment from day 4 to 7 to induce the colitis. Mice were intravenously administered with PBS or exosomes (60 μg/mouse) on days 2 and 4 after DSS drinking. Weight loss, stool consistency and rectal bleeding were monitored daily for 7 days and scored separately on scales of 0–4. The disease activity index (DAI) was calculated according to the average values of the three values. Colons were isolated and the lengths were measured. For the histological assessment, colons were fixed in 10% formalin solution, paraffin-embedded, sections and then stained with hematoxylin and eosin (H & E), the histological scoring was determined with a combined score for tissue damage (score, 0–3) and inflammatory cell infiltration (score, 0–3) as described previously (25).

Induction of T Cell Subsets

Naïve $CD4^+$ T cells were isolated using $CD4^+CD62L^+$T Cell Isolation Kit II (Miltenyi Biotec). Naïve $CD4^+$ T cells were cultured in a 24-well plate precoated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) mAbs under Th1, Th17, and Treg cell induction conditions for 3 days. Cytokines for T cell subset differentiation are as follows: Th1, IL-12 (5 ng/ml) and anti-IL-4 mAb (10 μg/ml); Th17, TGF-β (3 ng/ml), IL-6 (20 ng/ml), IL-23 (20 ng/ml), anti-IL-4 mAb (10 μg/ml) and IFN-γ mAb (10 μg/ml); Treg, TGF-β (2.5 ng/ml).

Statistical Analysis

The statistical significance was determined by the Student's t test or one-way, ANOVA. All analyses were performed using SPSS 16.0 software. P values <0.05 were considered statistically significant.

RESULTS

Characterization of OE-MSCs-Secreted Exosomes

OE-MSCs-Exos were isolated from the supernatants of OE-MSCs by ultracentrifugation method. Before the extraction of exosomes, the characteristics of OE-MSCs were identified. Several surface phenotypic markers were analyzed in OE-MSCs. As shown in **Figure 1A**, expression of CD29, CD90, and CD44 was positive on OE-MSCs, while CD34, CD45, and CD11b were negative. Furthermore, two neural stem cells-related markers, nestin and vimentin, were also expressed in OE-MSCs (**Figure 1B**). Likewise, OE-MSCs possessed the capacity to differentiate into adipocytes and osteocytes under indicated conditions (**Figure 1C**). All these data suggest that OE-MSCs display typical characteristics of MSCs and have been successfully purified.

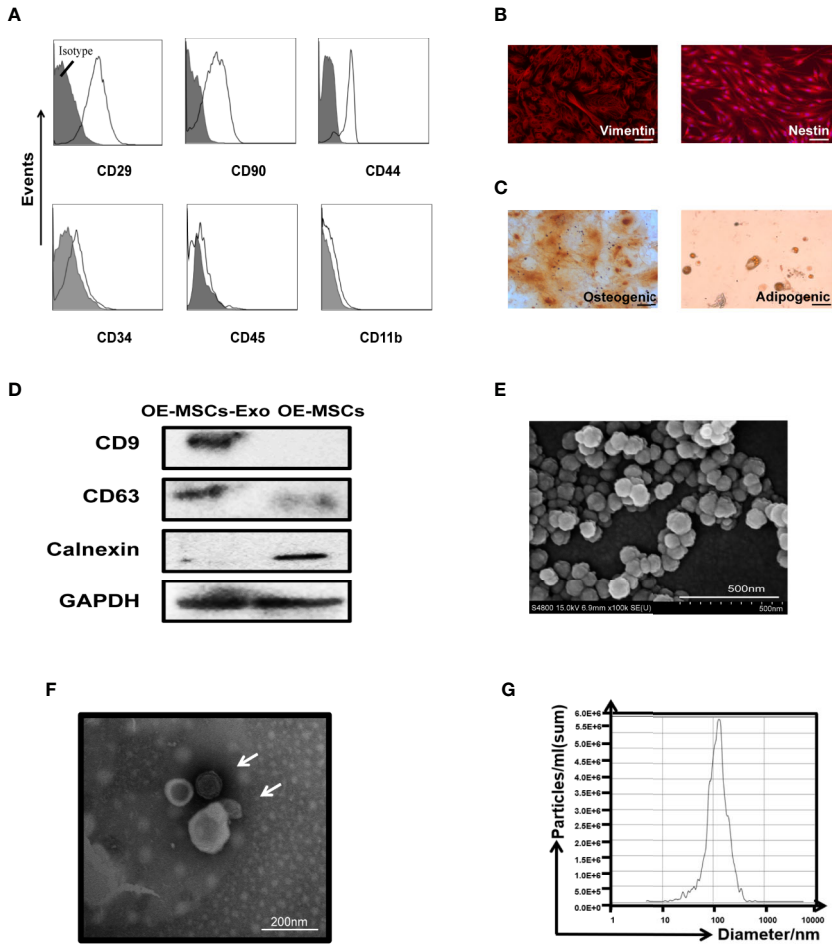

FIGURE 1 | Identification of OE-MSCs and exosomes released from OE-MSCs. **(A)** Immunophenotypes of OE-MSCs were detected by flow cytometry, including CD29, CD90, CD44, CD34, CD45, and CD11b. **(B)** Expression of vimentin and nestin (red) in OE-MSCs was detected by immunofluorescence with corresponding antibodies (original magnification ×200, bar = 30 μm). **(C)** OE-MSCs were cultured in osteogenic/adipocytes induction medium for 2/3 weeks, and the induced cells were then stained with oil red O/alizarin red, respectively (original magnification ×200, bar = 30 μm). **(D)** Western blot analysis was used to detect the CD9, CD63, and calnexin in OE-MSCs-Exos and OE-MSCs. **(E, F)** Representative SEM **(E)** (bar = 500nm) and TEM **(F)** (bar = 200 nm) micrographs of OE-MSCs-Exos. **(G)** Size profile of OE-MSCs-Exos was analyzed by NTA. Results are representative of three independent experiments.

Next, characteristics of exosomes were analyzed in OE-MSCs-Exos. Western blot analysis showed the isolated OE-MSCs-Exos displayed typical phenotypic features of Exos, they expressed CD63 and CD9, but not calnexin (**Figure 1D**). OE-MSCs-Exos exhibited a typical spheroidal shape surrounded by a double-layer membrane (**Figures 1E, F**), and the size distribution was also consistent with the exosomes (**Figure 1G**). Together, these data indicate the successful isolation and purification of OE-MSCs-Exos.

OE-MSCs-Exos Display Potent Immunosuppressive Effect on CD4$^+$T Cells

To investigate whether OE-MSCs-Exos possess any immunosuppressive function, CD4$^+$ T cells were stimulated with anti-CD3 mAb and anti-CD28 mAb in the presence of different concentrations of OE-MSCs-Exos. As shown in **Figure 2A**, the proliferation of CD4$^+$T cells was inhibited in a dose-dependent manner. Moreover, levels of IFN-γ and IL-17 were reduced in the supernatant of OE-MSCs-Exos treated group, but the concentrations of several of suppressive cytokines, such as TGF-β and IL-10, were significantly increased (**Figure 2B**).

Adoptive Transfer of OE-MSCs-Exos Attenuates the Severity of DSS-Induced Colitis

To evaluate the potential protective effect of OE-MSCs-Exos in DSS-induced IBD, OE-MSCs-Exos were administered to mice on days 2 and 4 after the colitis induction (**Figure 3A**). As shown in **Figure 3B**, the colons of the control group were obviously shortened and contracted when compared to the naïve mouse, whereas OE-MSCs-Exos treatment significantly increased the colon length and ameliorated the inflammation. Simultaneously, mice in the control group displayed remarkably elevated disease

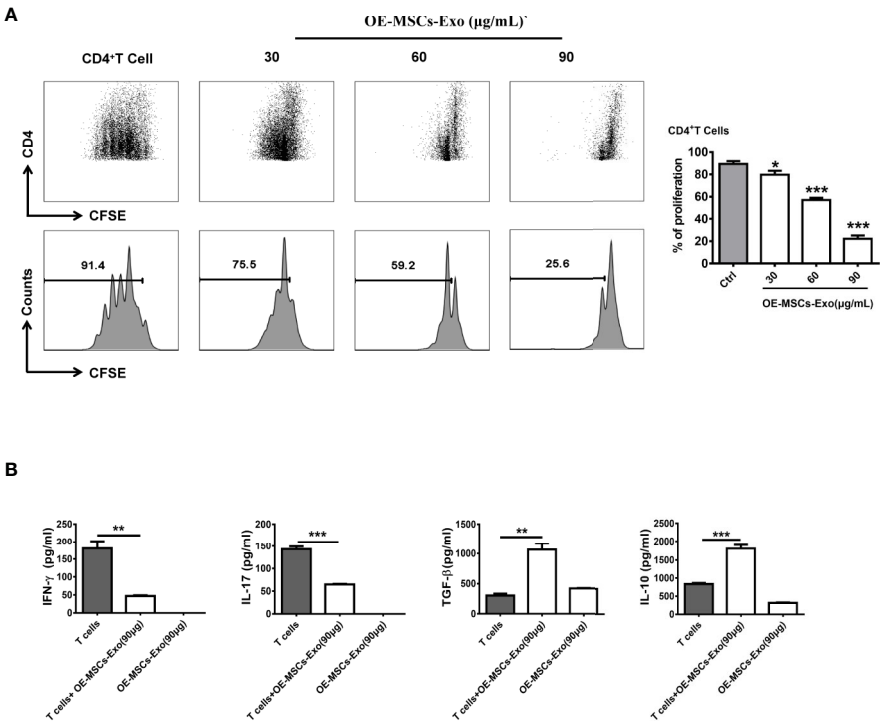

FIGURE 2 | OE-MSCs-Exos suppress the CD4$^+$T cell proliferation *in vitro*. **(A)** CFSE-labeled CD4$^+$T cells were treated with OE-MSCs-Exos (30, 60, 90 μg/ml) under CD3 and CD28 stimulation. After 72 h, the T cell proliferation was determined by flow cytometric analysis of CFSE dilution. **(B)** Levels of IFN-γ, IL-17, TGF-β, and IL-10 were measured by ELISA in the supernatants of CD4$^+$T cells with or without OE-MSCs-Exos (90 μg) and the lysis supernatant of OE-MSCs-Exos. Data are shown as mean ± SD from three independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

activity index (DAI). However, after the intravenous injection of OE-MSCs-Exos, the disease activity of mice was significantly decreased (**Figure 3C**). Furthermore, the histological examination showed that OE-MSCs-Exos treatment effectively retained the integral structure of the colon, with the reduced crypt loss, less lymphocytic infiltration, and lower histological scores when compared with the control group (**Figures 3D, E**).

OE-MSCs-Exos Reduced Th1/Th17 Responses and Enhanced Treg Expansion in Murine Experimental Colitis

It has been acknowledged that Th1 and Th17 play important roles in the pathogenesis of IBD, and Treg cells exert potential anti-inflammatory function to protect against the development of IBD (26). Thereafter, the proportions of Th1, Th17, and Treg cells were analyzed in mice with experimental colitis from different groups. As shown in **Figures 4A, B**, the IBD mice displayed significant increased Th1 and Th17 cell responses when compared to the naïve mice. After the treatment of OE-MSCs-Exos, the percentages of Th1 and Th17 cells in mesenteric lymph nodes (MLNs) were remarkably reduced. Simultaneously, the frequency of Treg cells in MLNs was obviously enhanced after OE-MSCs-Exos administration (**Figure 4C**). Together, OE-MSCs-Exos can efficiently inhibit Th1 and Th17 cells and increase Treg cells in experimental colitis.

OE-MSCs-Exos Inhibit the Differentiation of Th1/Th17 Cells but Promote the Treg Induction

Having observed the modulation of OE-MSCs-Exos in Th1, Th17, and Treg cells *in vivo*, we next established the differentiation system of Th1, Th17, and Treg *in vitro*, and evaluated whether OE-MSCs-Exos could affect the differentiation of these Th subsets directly, thus revealing the underlying mechanism of OE-MSCs-Exos in treating experimental colitis. As expected, under the Th1, Th17, and Treg differentiation conditions, OE-MSCs-Exos treatment could significantly inhibit the differentiation of Th1 and Th17 cells (**Figures 5A, B**), but promote the differentiation of Treg cells in a dose dependent manner (**Figure 5C**).

DISCUSSION

In this study, we first showed the typical MSCs phenotypic markers on OE-MSCs, and the capability of differentiating into osteocytes and adipocytes further confirmed they were of stem cell property. Moreover, the same as OE-MSCs, exosomes released by OE-MSCs were also demonstrated to possess immunosuppressive function, presented as the inhibition of CD4$^+$ T cell proliferation, decreased IFN-γ, IL-17, and

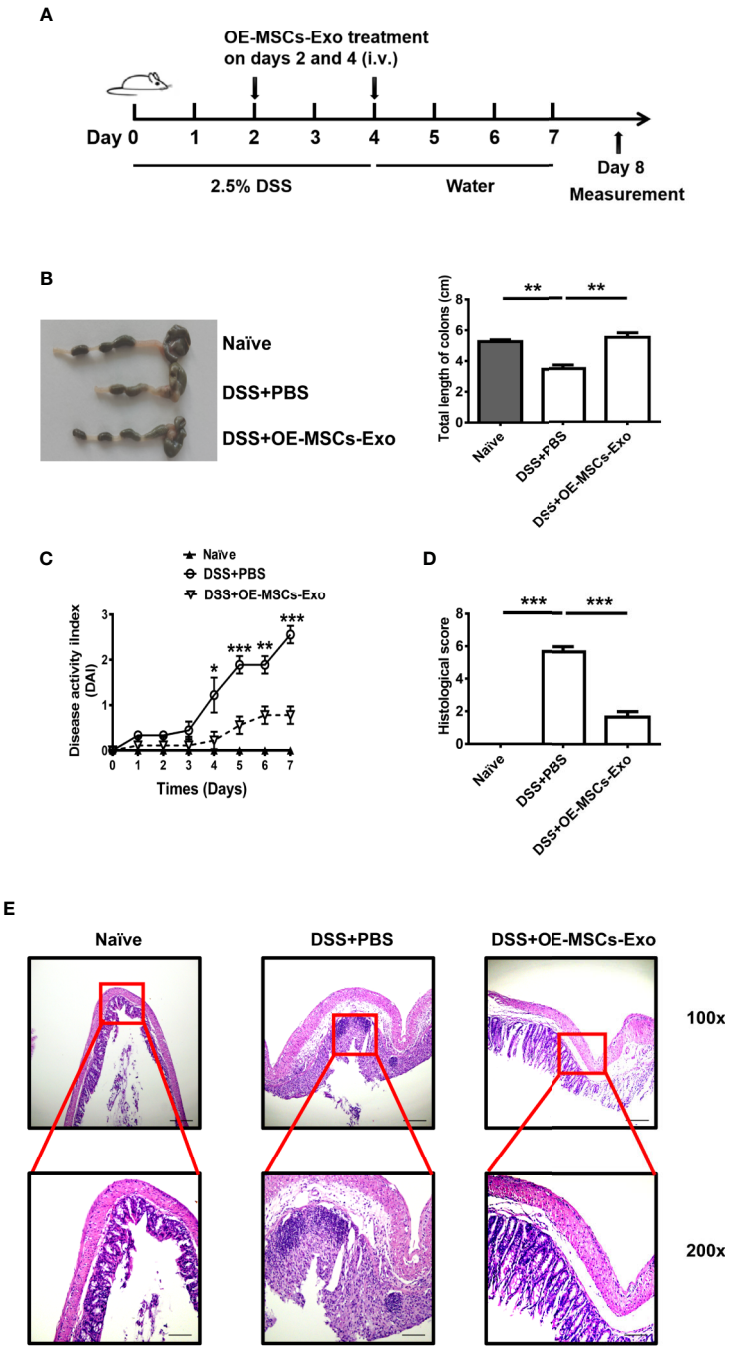

FIGURE 3 | OE-MSCs-Exos protect against the murine experimental colitis. **(A)** Graphic scheme of DSS-induced colitis induction and OE-MSCs-Exos administration. C57BL/6 mice were subjected to 2.5% DSS in the drinking water for 4 days, followed by water treatment from day 4 to 7. OE-MSCs-Exos (60 μg/mouse) were i.v. injected into the mice on days 2 and 4 (n = 6/group). **(B)** Mice were sacrificed on day 8, the length of colons was measured. **(C)** DAI scores were evaluated and recorded everyday. **(D, E)** The histopathological detection of the colon sections was analyzed by H&E staining **(E)**, and the histopathological scores were determined **(D)**. Original magnification ×100 (upper, bar = 150 μm), ×200 (lower, bar = 50 μm). Data are shown as mean ± SD from three independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

enhanced TGF-β, IL-10. Afterwards, adoptive transfer of OE-MSCs-Exos exhibited obvious therapeutic effect in the experimental colitis mice, both Th1 and Th17 cell responses were suppressed while Treg cells were expanded after OE-MSCs-Exos treatment. Furthermore, the *in vitro* data clarified that OE-MSCs-Exos could directly inhibit the differentiation of Th1/Th17 cells whereas promote the Treg induction. Taken together, OE-MSCs-Exos exerted their immunoregulation and effectively

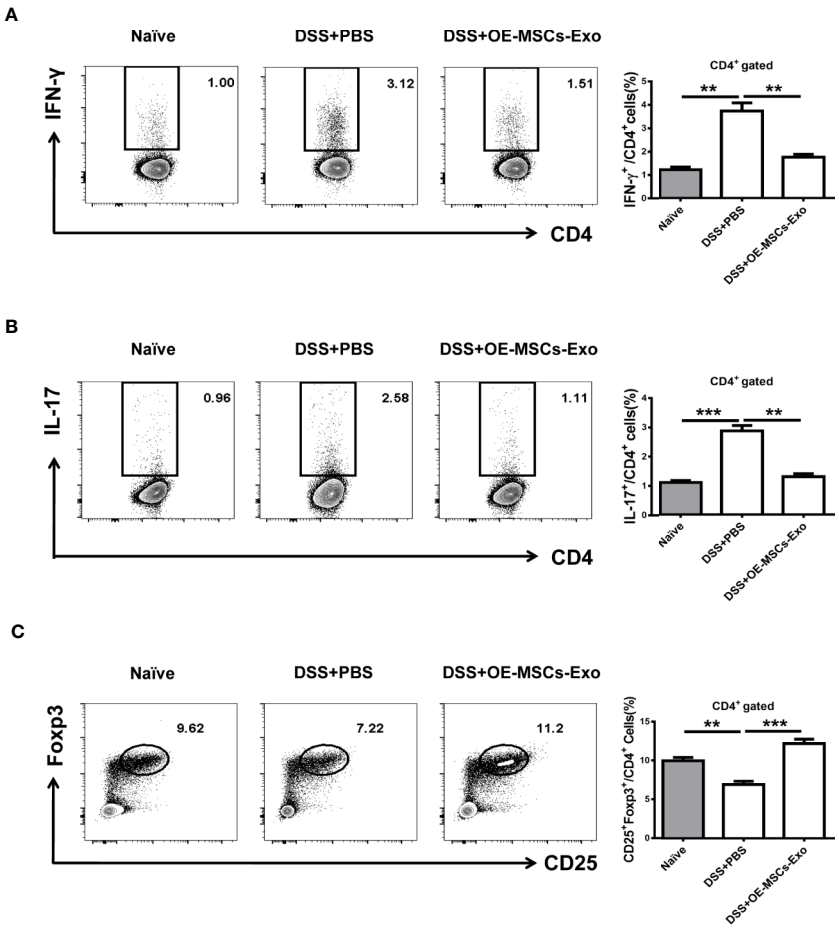

FIGURE 4 | OE-MSCs-Exos reduce Th1/Th17 cells and expand Tregs in experimental colitis. C57BL/6 mice were subjected to 2.5% DSS in the drinking water for 4 days, followed by water treatment from day 4 to 7, and OE-MSCs-Exos (60 μg/mouse) were i.v. injected into the mice on days 2 and 4. Mice were sacrificed on day 8 (n = 6/group). **(A–C)** Proportions of Th1 **(A)**, Th17 **(B)**, and Treg **(C)** cells in MLNs were measured in each group by FCM. Data are shown as mean ± SD from three independent experiments. ***$p < 0.001$, **$p < 0.01$.

alleviated the disease severity in experimental colitis *via* regulating T cell responses.

MSCs have been reported to be found in various tissues, such as bone marrow, muscle, adipose, umbilical cord, placenta and so on (27, 28). Despite the sources are different, MSCs mostly share a similar characteristic phenotype. As we shown, OE-MSCs was a population of stem cells originate from olfactory lamina propria, possessing the typical characteristics of stem cells. Due to the immunosuppressive function of MSCs, they have been widely applicated in various autoimmune diseases and inflammatory diseases. Our previous data have demonstrated that OE-MSCs displayed stronger immunosuppressive capacity than BM-MSCs in regulating T cell responses (21). Recent years, exosomes released by MSCs have emerged as a novel and powerful secretory component of MSCs and aroused great interest. MSCs exert their immunologic properties mainly by paracrine pathway, and exosomes are supposed to be an essential manner in the process (29). Thus, the immunoregulation and anti-inflammatory properties of MSCs are possibly found in MSC-derived exosomes. Indeed, several studies have identified the immunomodulatory properties of MSC-derived exosomes *in vitro* and animal models (16, 29, 30). In our study, exosomes derived from OE-MSCs were demonstrated to possess immunosuppressive function in regulating $CD4^+$ T cell proliferation, and levels of inflammatory cytokines IL-17 and IFN-γ in OE-MSCs-Exos-treated supernatant were decreased, while the inhibitory cytokines TGF-β and IL-10 were increased, suggesting OE-MSCs-Exos might exert their immunoregulation function by suppressing the effector T cells and enhancing regulatory T cells.

MSC-Exos-based therapy is more than a compensation for MSC-based treatment. Exosomes have several potential advantages over the corresponding MSCs: first, the smaller size of exosomes can improve the therapeutic delivery to the desired sites by reducing the entrapment in capillaries after systemic administration. Also, the small size and less complicated

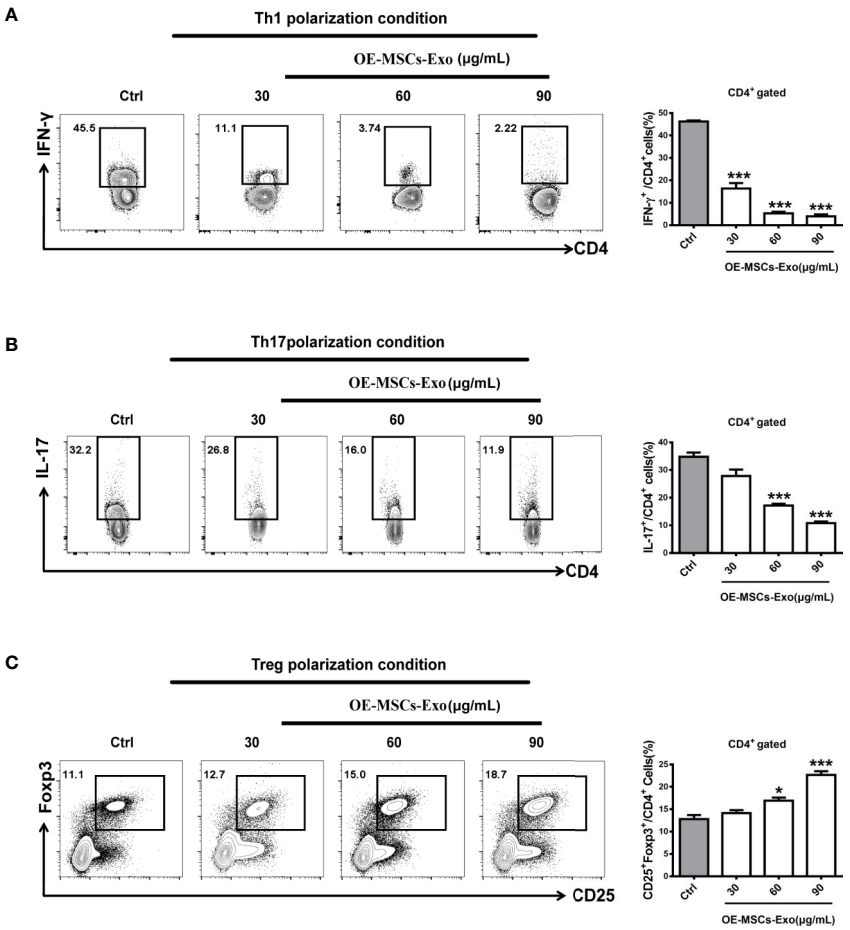

FIGURE 5 | OE-MSCs-Exos suppress Th1 and Th17 induction while promote Treg differentiation. **(A–C)** 1×10^6/ml naïve $CD4^+$ T cells were purified from the spleen of C57BL/6 mice and cultured for 72 h in the presence of anti-CD3 and anti-CD28 mAbs under Th1 **(A)**, Th17 **(B)**, or Treg **(C)** differentiation conditions, respectively. Different concentrations of OE-MSCs-Exos (30, 60, 90 μg/ml) were added into each differentiation system. Frequencies of Th1, Th2, and Th17 cells were detected by FCM. Data are shown as mean ± SD from three independent experiments. ***$p < 0.001$, *$p < 0.05$.

structure are relatively easier for storage and production. Second, due to their acellular status, exosomes will have lower side effects and other risks induced by cells, such as less vascular obstructive propensity. In addition, the acellular status will protect exosomes from being modified by the *in vivo* environment, thus showing higher stability compared to cells. Besides, the properties of non-oncogenicity, immunosilence and tissue-specific homing allow MSC-Exos to be an ideal therapeutic tool in a variety of diseases (12, 29), including IBD. Although the etiology of IBD is unclear, dysregulated immune responses have been considered to be involved in the pathogenesis of IBD. In patients with IBD, percentages of effector T helper (Th) subsets, including Th1 and Th17 cell populations, were significantly increased in peripheral circulation. However, the proportion of Treg cells was showed to decrease (31, 32). The imbalance of effector Th cells and Tregs is supposed to be an essential element in the development of IBD. MSCs are known to regulate both innate and adaptive immune responses *via* secreting immunomodulatory factors and control the inflammation in IBD. As we previously reported, OE-MSCs possess several advantages when compared to BM-MSCs. Nasal lamina propria is the tissue easily to harvest and OE-MSCs can used for autologous transplantation. Furthermore, OE-MSCs exhibited higher proliferation profile and stronger suppressive capacity than BM-MSCs (20, 21, 33). Accumulating evidence have clarified exosomes can carry various mRNAs, microRNAs, and proteins from MSCs, and function as an extension of MSC's biological role. Thus, considering the above advantages, exosomes released by OE-MSCs may serve as a potential cell-free therapy for IBD. As expected, adoptively transfer of OE-MSCs-Exos could efficiently ameliorate the severity of mice with experimental colitis, presented as reduced disease activity, less lymphocytic infiltration. Concurrently, Th1 and Th17 subpopulations were remarkably reduced whereas Treg cell increased in MLNs after OE-MSCs-Exos treatment. Mechanistically, OE-MSCs-Exos were demonstrated to inhibit the differentiation of Th1 and Th17 cells,

but promote the induction of Treg cells *in vitro*. It has been acknowledged that MSCs-Exos can carry various regulatory molecules, such as IL-10, TGF-β, PGE2, IDO, PD-L1, and Gal-1 (34). However, the possible molecular mechanism for OE-MSCs-Exos to regulate the differentiation of T cell subsets still needs further exploration.

In summary, we have identified the immunoregulatory property of exosomes derived from OE-MSCs. Furthermore, OE-MSCs-Exos could exert their immunomodulation capacity to ameliorate disease severity in IBD mice, mainly by regulating Th-cell immune responses. These findings suggest that OE-MSCs-Exos represent a novel potential cell-free therapy for targeting inflammatory diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on the Use of Live Animals in Research and Teaching of Jiangsu University.

AUTHOR CONTRIBUTIONS

JT performed the experiments, analyzed the data, and wrote the paper. QZ, YZ, QB, and ZS performed the experiments. YH, HX, and KY analyzed the data. SW and KR designed the study and wrote the paper. All authors contributed to the article and approved the submitted version.

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Extracellular Vesicles in Rheumatoid Arthritis and Systemic Lupus Erythematosus: Functions and Applications

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In the last two decades, extracellular vesicles (EVs) have aroused wide interest among researchers in basic and clinical research. EVs, small membrane vesicles are released by almost all kinds of cells into the extracellular environment. According to many recent studies, EVs participate in immunomodulation and play an important role in the pathogenesis of autoimmune diseases. In addition, EVs have great potential in the diagnosis and therapy of autoimmune diseases. Here, we reviewed the latest research advances on the functions and mechanisms of EVs and their roles in the pathogenesis, diagnosis, and treatment of rheumatoid arthritis and systemic lupus erythematosus.

Keywords: extracellular vesicles, exosome, systemic lupus erythematosus, microRNA, rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are both autoimmune diseases that can involve multiple organs. Their etiologies and pathogenesis are complex, and epigenetic and environmental factors are shown to be associated with the onset of the disease (1, 2). Glucocorticoids, immunosuppressants, and biological agents are commonly used in the treatment of RA and SLE, but problems such as toxic side effects and non-response to treatment remain (3–5). EVs are phospholipid bilayer-enclosed vesicles secreted from all cell types. The classification of EVs includes exosomes (<150 nm), microvesicles (150–1,000 nm) (6), and apoptotic bodies (1,000–5,000 nm), depending on size and biogenesis (7). EVs play an important role in cellular communication processes. In the past, intercellular communication was thought to have two modes, direct contact between cells and secretion of cellular molecules (8). The relationship between EVs and cellular communication has attracted more attention and has become the third mechanism of intercellular communication (9). EVs began to be isolated and studied from additional cell types, such as immune cells, nerve cells and tumor cells (10). It is demonstrated that EVs are involved as carriers in intercellular communication by transporting lipids, proteins, and other components (11). In 1996, Raposo et al. first showed that EVs could stimulate adaptive immune responses (12). EVs can also carry mitochondria to regulate immunity and alter the phenotype of macrophages (13).

EVs are secreted by almost every functional cell type and have the characteristics of easy detection and stability. Body fluids, such as urine and peripheral blood contain EVs, which present promising prospects as biomarkers for tumors, infectious diseases, and autoimmune diseases. Furthermore, the biological characteristics of EVs that can transport multiple cellular components also make it possible to use them in therapeutic approaches for diseases. To date, there have been some studies of EV treatment for RA and SLE, which have made certain achievements (14, 15). Here, we summarize the functions of EVs on immune cells and their applications in the pathogenesis, diagnosis and treatment of RA and SLE.

THE BIOGENESIS AND COMPOSITION OF EVS

Different types of EVs have slightly different biological origins, and their biological functions are determined by their respective intercellular components. Exosomes are EVs with a diameter of no more than 150 nm. The limiting membrane of late endosomes generates exosomes by invagination and budding (16). Then, exosomes are covered by endosomal multivesicular bodies (MVBs) and form intraluminal vesicles (ILVs), which fuse with the plasma membrane and are exocrine. The endosomal sorting complex required for transport (ESCRT) is also involved in exosome generation, which is formed by approximately twenty proteins (17). The action of ESCRT is mainly carried out by four proteins following specific steps. First, ESCRT-0 recognizes ubiquitinated proteins in the endosomal membrane and isolates them individually. Second, both ESCRT-I and ESCRT-II mediate the transformation and assembly of the membrane. Third, ESCRT-III leads to the scission (18). Exosomes contain proteins, nucleic acids, lipids, and organelles such as mitochondria (19).

Microvesicles (MVs) are formed as the plasma membrane germinates outward directly. Although the diameter-based classification of exosomes and MVs is somewhat controversial, the fundamental distinction is apparent based on their biogenesis. Their formation is related to changes in the symmetry of phospholipids in cell membranes, and their release is associated with lipid rafts on the cell membrane (20). Proteins and phospholipids are unevenly distributed on the plasma membrane by the regulation of aminophospholipid translocases. The transfer of phosphatidylserine and the change in protein structure create a dynamic equilibrium, contributing to the formation of MVs (21). MVs are composed similarly to exosomes.

Apoptotic bodies are the products of apoptosis, while exosomes and MVs are secreted by living cells. The contents of the cell after apoptosis decompose into membrane-bound vesicles. In terms of composition, apoptotic bodies are characterized by the inclusion of organelles and smaller vesicles (21). Apoptotic bodies also contain ribosomal RNA which are almost undetectable in exosomes and MVs (22). They work primarily as garbage carriers of cells containing cellular wastes (23).

THE ROLE AND MECHANISM OF EVS IN THE IMMUNE SYSTEM

Research on EVs began in 1983 when exosomes were first identified in reticulocytes from sheep (24). However, it was not until 1996 that B cells were shown to release exosomes with the major histocompatibility complex class II (MHC II), which indicated the relationship between immune cell regulation and exosomes (12). Other immunocytes, such as T cells, natural killer (NK) cells, and dendritic cells (DCs), have been proven to be associated with EVs in recent publications (25–27). Since EVs, especially exosomes, can carry MHC II, it is possible for EVs to participate in antigen presentation. Tian et al. summarized three mechanisms by which EVs are involved in antigen presentation (7). First, loading antigen proteins inside exosomes improves the efficiency of antigen presentation, and then APCs costimulating molecules act on the activation of T cells (28). Second, when peptide/MHC complexes are formed, exosomes with antigens can be captured by APCs and then are exposed to the cell membrane to activate T cells. Third, EVs directly activate T cells without the participation of APCs (29). Interestingly, reverse transport of miRNA by exosomes, which is antigen-driven, has been proven to regulate the gene expression of APCs (30).

DCs are one of the most effective immunocytes in presenting antigens and are critical to both innate and adaptive immunity. Some DCs can establish immune tolerance by reducing the T cell activity level, while the other DCs can activate T cells to enhance the immune response. With the expression of a high level of MHC I/peptide complexes as well as B7 and ICAM-1, exosomes from DCs are able to directly activate $CD8^+$ T cells without the participation of normal APCs (31). Not only the mutual effect between DCs and T cells but also the intercellular communication between DCs play crucial roles in the process of DCs regulating innate immune responses (32). Angela Montecalvo et al. found that the miRNA components of exosomes released by DCs at different stages of maturation were different. Mature DC-derived exosomes show a stronger T-cell stimulatory ability than immature DC-derived exosomes because of higher expression of CD86 and CD54 (32). DC-derived exosomes (Dexs) containing MHC/peptide complexes can boost T cell-dependent tumor rejection. And NK cells can be activated by both IL-15Ra and NKG2D ligands in Dexs and secrete IFN-γ (33).

Similar to other APCs, B cells have a cellular structure called the endocytic compartment MIIC (major histocompatibility complex [MHC] class II-enriched compartment), which participates in the activation of antigen-specific MHC II-restricted T cell responses (12). Activated B cells infected with EBV can also excrete exosomes with EBV-miRNAs, which accumulate in neighboring primary immature monocyte-derived DCs (MoDCs) without infection (34). Furthermore, exosomes from activated B cells with EBV infection harbor the viral latent membrane protein 1 (LMP1), which imitates CD40 signaling, resulting in the propagation of B cells as well as T cell-independent class-switch recombination (35).

NK cells are important immunocytes in innate immunity with a variety of biological functions, including recognizing and killing viral infections and tumor cells, and producing cytokines

such as interferon (IFN)-γ, involved in immune regulation (36). After being activated by Dexs, NK cells secrete exosomes containing CD63, fibronectin, perforin, granulysin, and granzyme A/B, which indicates that NK-derived EVs contain the killing function of NK cells (26). The EV interaction between APCs and T cells as well as NK cells is shown in **Figure 1**.

Other non-immune cells can also produce EVs and participate in the regulation of immune responses. EB virus (EBV) infected cells can secrete exosomes containing EBV-microRNAs (miRNA) to mediate gene silencing in immune cells (37). EVs derived from tumor cells and stem cells have also been shown to regulate immune function (38). miRNAs in tumor exosomes may induce immune tolerance (39). While EVs derived from stem cells have been shown to regulate immunity and reduce inflammatory responses (40, 41). Lipid-filled vesicles, derived by adipocytes, can modulate tissue macrophage to participate in immune regulation (42).

THE ROLE OF EVS IN RA

EVs Are Involved in the Pathogenesis of RA

RA is an autoimmune disease with a high incidence that damages multiple joints throughout the body and can cause progressive disability. RA is characterized by synovial inflammation and cartilage destruction (43). In comparison with those from normal controls, EVs showed a high density in the synovial fluid of RA samples, which was associated with disease progression (44). EVs are mainly involved in antigen presentation, inflammatory cytokine and miRNA transmission, and activation of fibroblast-like synoviocytes (FLSs) in the pathological process of RA. It has also been shown that FLSs-derived EVs contained hexosaminidase D activities in the samples of RA patients (45). Additionally, the level of N-acetyl-beta-D-hexosaminidase (NAHase) in destructive RA is higher than that in inflammatory RA, indicating that glycosaminoglycan-degrading glycosidases may cause joint damage in RA (46). Citrullinated proteins can be detected in synovial exosomes, which can enhance T cell activity with fibronectin (47). In addition, antibodies to citrullinated protein antigens (ACPAs) are crucial in the pathological process of RA and are expected to work as biomarkers with the highest predictive value (48, 49).

FLS-derived microparticles (MPs) contain B cell stimulation factors in the synovial fluid of the joints of RA patients (50). There are microparticle-containing immune complexes (mpICs) in synovial fluid with CD41 highly expression, indicating platelet-derived. These mpICs could induce neutrophils to release leukotrienes, which proves that platelet mpICs are proinflammatory and highly reactive (51). Also, platelet-derived microparticles (MPs) seem to release IL-1β, which promotes joint inflammation by increasing the levels of IL-6 and IL-8 in fibroblasts from RA patients (52). Tumor necrosis factor-α (TNF-α) is crucial to the pathogenesis of RA. TNF-α binding membranes were detected in FLSs-derived EVs from RA patients. EVs containing TNF-α activated AKT and NF-κB and rendered these activated T cells resistant to apoptosis (53). Additionally, T cell-derived MPs treated with TNF-α could upregulate prostaglandin E2 (PGE2), microsomal prostaglandin E synthase 1 (mPGES-1) and cyclooxygenase 2 (COX-2) (54). Then COX2

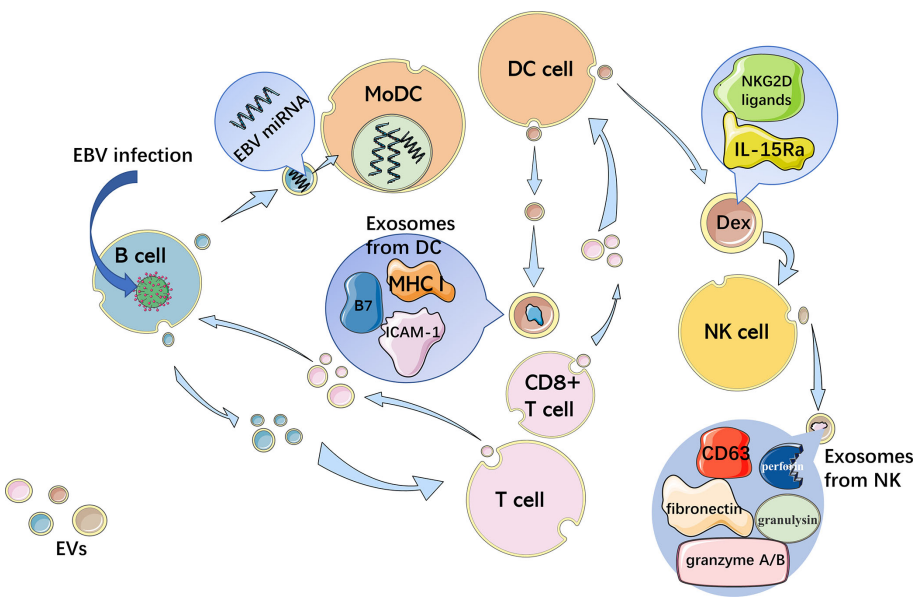

FIGURE 1 | Interaction between immune cells through EVs. APCs activate T cells with EVs and reverse transport of miRNA by exosomes, which is antigen-driven, regulating the gene expression of APCs. B cells infected with EBV excrete exosomes with EBV-miRNAs, accumulating in neighboring MoDCs. Exosomes with MHC I/peptide complexes, B7 and ICAM-1 from DCs, directly activate CD8+ T cells. Dexs containing IL-15Ra and NKG2D ligands activate NK cells, which then secrete exosomes with CD63, fibronectin, perforin, granulysin, and granzyme A/B.

caused pain and inflammation in patients. Coinhibitory T cell receptors can be expressed in cells from RA joints, including PD-1 and TIM-3. EVs from synovial fluid and T cells after cocultivation could express PD-1. Carrying the PD-1 receptor and inhibitive miRNAs, EVs may induce T cell exhaustion (55).

The transmission of miRNAs is crucial in the RA pathological process. Of all the miRNAs associated with RA, miR-155 and miR-146a have attracted most attention. A study proved that exosomal miR-146a and miR-155 are expressed at high levels in RA synovial tissue (56). Furthermore, miR-155 can be upregulated by stimulation with proinflammatory mediators, including Toll-like receptor (TLR) ligands, TNFα and IL-1β. Overexpression of miR-155 in RA synovial fibroblasts (RASFs) can downregulate matrix metalloproteinase 3 (MMP-3) and MMP-1 (57). MMP-3 is involved in the generation of severe cartilage damage (58). All these components in EVs contribute to the onset and development of RA (**Table 1**).

The Role of EVs in Diagnosis and Treatment of RA

Existing studies implied that EVs have potential as biomarkers for RA. RA patients with IgM-rheumatoid factor (RF) EVs showed high-level C-reactive protein (CRP) and Erythrocyte sedimentation rate (ESR) levels compared with those of RA patients without IgM-RF in EVs (63). Thus, EVs with IgM-RF can be used to distinguish between active and inactive RA. RA patients express high levels of MPs in the circulatory system compared with those in healthy controls (64). Moreover, as the role of miRNA in the pathology of RA disease has been revealed, exosomal miRNAs, including miR-155 and miR-146a, can be used for the early diagnosis of RA (57). Potential biomarkers for RA in EVs are summarized in **Table 2**.

Mesenchymal stem cells (MSCs) have anti-fibrosis and anti-inflammatory immune regulatory effects. The transplantation of MSCs has been used as a new technique for RA therapy. When collagen-induced arthritis (CIA) rats were treated with human umbilical cord MSCs (hUCMSCs), the results showed that hUCMSCs can reduce T lymphocyte activity and function, as well as inhibit Th17 cells and induce Treg cells to alleviate the disease (78). The same immunomodulatory function between hUCMSCs and hUCMSC-derived EVs has been demonstrated *in vitro*, which indicates the potential of hUCMSC-derived EVs as a new treatment for RA (78). hUCMSC-derived EVs also can inhibit the expression of IL-17 by downregulating Th17 cells and increasing the proportion of Treg cells in a dose-dependent manner. Moreover, it was demonstrated that periarticular injection of exosomes containing IL-10 or exosomes from bone marrow-derived DCs could relieve arthritis by anti-inflammatory action since DC-derived exosomes showed strong anti-inflammatory and immunosuppressive activity through the class II-dependent pathway. In addition, as exosomes are phenotypically stable after purification *in vitro* (79), EVs could potentially be a drug carrier for precise therapy for RA. Louise et al. used the human neutrophil-derived EVs as scaffolds, which have the function of immune regulation and cartilage penetration. Anti-reactive oxygen species-collagen type II (Anti-ROS-CII) is an antibody targeting impaired arthritic cartilage. Combining this antibody with EVs allows the complex to penetrate the cartilage into the articular cavity and still maintain antibody activity, suggesting the potential of EVs as a targeted carrier for drug delivery (80).

TABLE 1 | EVs involved in the pathogenesis of RA and SLE.

Disease	Molecular/Parameter	Reference
RA	Density of EVs	(44)
	FLSs-derived EVs contained hexosaminidase D	(45)
	Citrullinated proteins in exosomes	(47)
	Microparticles-containing immune complexes	(51)
	Platelet-derived Microparticles	(52)
	TNF-α contained in EVs	(53)
	Exosomal miR-155	(57)
	Exosomal miR-146a	(8)
SLE	Exosomal miR-146a	(59)
	Exosomal miR-21	(60)
	Exosomal miR-574	(61)
	MVs from apoptosis	(62)

THE ROLE OF EVS IN SLE

EVs Are Involved in the Pathogenesis of SLE

SLE is a complex heterogenous autoimmune disease that involves damage to multiple organs throughout the body and can cause death in severe cases. Patients with SLE are characterized by T and B lymphocyte dysfunction, accumulation of autoantibodies, and deposition of immune complexes (81). However, the pathogenesis of SLE remains unclear. The role of EVs in the pathogenesis of SLE is of interest to researchers.

Exosomal miRNAs in exosomes play an important role in the development of SLE. The level of miR-146a contained within exosomes in the urine of lupus patients was significantly higher than that outside of exosomes. In contrast, miR-146a levels in serum exosomes were significantly lower in SLE patients than in HCs (59). Of all miRNAs, miR-146a can significantly distinguish active LN from inactive LN and is related to inflammation and fibrosis of the kidney (74). In addition, miR-146a may be upregulated by chemokines as well as proinflammatory cytokines and leads to anemia in SLE patients (82). MSCs can internalize exosomes with miR-146a and target TRAF6/NF-κB signaling, leading to the senescence of MSCs (59). The senescence of MSCs may be related to the disease activity and pathological process of SLE (83, 84). Another important exosomal miRNA is miR-21 contained in EVs, facilitating estrogen-regulated STAT1 activation and Toll-like receptor (TLR) 8 expression in SLE. miRNAs can be endogenous ligands of human TLR7, which is the single-stranded RNA (ssRNA) receptor expressed by plasmacytoid dendritic cells (pDCs). miR-21 can replace viral ssRNA to combine with TLR8 to stimulate innate immune responses (60). Interferon (IFN)-α plays a major role in SLE (85). It was proven that miRNAs in exosomes, such as miR-574, upregulated type I IFNs secreted by pDCs in SLE (61). MVs from apoptosis in SLE serum can activate cyclic guanosine monophosphate (GMP)-AMP

TABLE 2 | EVs as potential biomarkers for RA and SLE.

Disease	Molecular	Change	Body fluids	Reference
RA	$IgMRF^+$ EVs	Positive correlation with disease activity	Serum	(65)
	$CD41^+$EVs	Upregulated	Synovial fluid	(44)
	$CD3^+CD8^+$Tcell-derived EVs	Upregulated	Synovial fluid	(66)
	Hotair in EVs	Upregulated	Serum/urine	(67)
	Exosomal miR-106b	Upregulated	Synovial fluid	(68)
	Exosomal miR-6089	Downregulated	Serum	(69)
	Exosomal miR-548a-3p	Downregulated	Serum	(70)
	Citrullinated proteins in EVs	Upregulated	Synovial fluid	(47)
	miR-146a and miR-155	Upregulated	Synovial fluid /plasma	(71)
SLE	Urinary exosomal miR-135b-5p, miR-107, miR-31	Upregulated in LN	Urine	(72)
	Urinary exosomal miR-21, miR-150, and miR-29c	Correlated with LN chronicity index (CI)	Urine	(73)
	Exosomal miR-146a	Downregulated	Serum	(74)
	Urinary podocyte-derived MPs	Positively correlated with the SLE Disease Activity Index (SLEDAI) score	Urine	(75)
	Endothelial cells-MPs	Upregulated	Serum	(76)
	Urinary MP-HMGB1	Upregulated in active LN than inactive	Urine	(77)
	Monocytic CD 14^+ MP	Positively correlated with the disease activity in SLE	Plasma	(65)

synthase (cGAS), which stimulates the stimulator of interferon genes (STING) pathway and upregulates the type I IFN production (62). The EVs involved in the pathogenesis of SLE were shown in **Table 1**.

The Role of EVs in Diagnosis and Treatment of SLE

EVs can be used to measure disease activity and differential diagnosis in patients with LE. Damage to glomerular podocytes is crucial in renal injury in SLE. Urinary podocyte-derived MPs can be used for the prediction of disease activity. They are positively correlated with clinical indicators of SLE, including erythrocyte sedimentation rate, proteinuria, and SLE Disease Activity Index (SLEDAI) score (75). Urinary HMGB1 in MPs is expressed at a significantly high level in active LN, which can distinguish between active and non-active LN (77). And identification of MPs with different surface proteins in SLE patients can predict disease activity and vascular damage (86). It was reported in another study that high plasma expression of monocytic CD 14^+ MP has a positive correlation with the disease activity of SLE (65). Compared with healthy controls and systemic sclerosis (SSc) patients, SLE patients presented a higher expression of endothelial cell MP (EMP), suggesting that EMP has potential as a biomarker for SLE vascular lesions (76). Potential biomarkers for SLE in EVs are summarized in **Table 2**.

EVs have also received further attention in the treatment of SLE. In LN, MP surface proteins, especially G3BP, play a key role in the deposition of ICs. Therefore, targeting MPs may be a new approach for treating LN (87). MSC-derived MVs have anti-inflammatory and immunomodulatory effects (88). Although the use of MSCs in the treatment of SLE is mature and has been used clinically (89–93), Juhi et al. found that MSC-derived EVs can replace MSCs in the treatment of SLE, with the following advantages. First, there is no evidence that EVs are carcinogenic. Second, compared with MSCs, EVs are more stable and easier to preserve in the long term. Third, EVs do not cause an immune response that harms the host. EVs can bypass the blood–brain barrier, which makes it possible for EVs to be used in the treatment of lupus encephalopathy (94). In addition, EVs are easier to prepare on a large scale and at a low cost for clinical therapy. However, the effect of EVs is closely related to the dose, and the appropriate therapeutic dose needs to be explored.

CONCLUSION AND OUTLOOK

Although EVs were discovered in 1983, research on EVs has grown rapidly only in the current century. The role of EVs in cellular communication and immune regulation is being gradually explained. EVs secreted by immune cells are involved in antigen presentation and regulation of immunity. Cytokines or miRNAs contained in EVs and MSC-derived EVs play important roles in autoimmune diseases. Technology for isolating and purifying EVs is growing (95–98). New technologies, such as nanoscale flow cytometry (NanoFCM) and microfluidic platforms with 100,000 pillars, have been used for more efficient isolation of EVs (99, 100). A microfluidic cell culture platform using a 3D-printed microfluidic chip has also been used in the preparation of EVs (101). The research development of EVs is helpful to understand the pathogenesis of autoimmune diseases and provide new ideas for diagnosis and treatment. At the same time, we should also pay attention to the role of EVs in the onset and development of diseases and emphasize the dose and safety in the treatment to avoid potential side effects.

AUTHOR CONTRIBUTIONS

BZ wrote the manuscript. MZ and QL conceptualized and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Neutrophil Extracellular Traps Tied to Rheumatoid Arthritis: Points to Ponder

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In recent years, neutrophil extracellular traps at the forefront of neutrophil biology have proven to help capture and kill pathogens involved in the inflammatory process. There is growing evidence that persistent neutrophil extracellular traps drive the pathogenesis of autoimmune diseases. In this paper, we summarize the potential of neutrophil extracellular traps to drive the pathogenesis of rheumatoid arthritis and experimental animal models. We also describe the diagnosis and treatment of rheumatoid arthritis in association with neutrophil extracellular traps.

Keywords: neutrophil extracellular traps, citrullinated autoantigens, anti-citrullinated protein antibodies, autoimmunity, chronic inflammation, protein-arginine deiminase type 4, rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease with high disability and increased mortality. It is characterized by progressive joint damage and synovial membrane hypertrophy. There are stark differences in the prevalence among different ethnicities and populations. RA is a heavy burden for the patients, their families, and society. To date, studies have shown that RA is a multifactorial disease involving age, sex, environmental, epigenetic, and genetic factors. However, the pathogenesis of RA is not fully understood (1). Many studies have shown that both innate immune response and adaptive immune response contribute to the etiopathogenesis of RA (2). It's considered that the formation of autoantibodies to citrullinated antigens (ACPA) is a critical pathogenic event involved in the development of RA. Neutrophils isolated from patients suffering from autoimmune diseases present enhanced formation of neutrophil extracellular traps (NETs). The role of neutrophils in autoimmune disease is still elusive (3). The release of cytotoxic products [e.g., degradation enzymes and reactive oxygen species (ROS)] from activated neutrophils into the synovial fluid and pannus in RA has been known for a long time and is considered important for RA (4, 5). In recent years, it was discovered that neutrophils participate in the inflammatory progression of RA through multiple regulatory immune mechanisms, including directly secreting cytokines and chemokines, and releasing neutrophil granules that activate or inactivate cytokines and chemokines. Enzymes upregulate the expression of MHC II and promote cell-cell interactions.

A novel role of neutrophils, the release of NETs, has attracted increasing attention. Upon pharmacological activation with phorbol myristate acetate (PMA) (6), interleukin 8 (IL-8) (7), or lipopolysaccharide (LPS) (8), neutrophils release granule proteins and chromatin to form NETs. The release of NETs (9) constitutes a novel programmed cell death that differs from apoptosis (10). LPS-induced NET formation increases with adhesion and substrate elasticity, while PMA-induced

NET formation is independent of adhesion (6). NETs are composed of chromatin and granular proteins, which trap and kill bacteria (11, 12). Most DNA is derived from nuclei however, mitochondrial DNA is also included. The proteins consist of neutrophil elastase (NE), myeloperoxidase (MPO), histones, defensins, calprotectin (13), matrix metalloproteinase-9, and others (14). During NET formation, NE, MPO, and peptidyl arginine deiminase (PAD4) promote nuclear decondensation and histone citrullination, respectively (15). NETs can be quantified and analyzed by DNA area and NETosis analysis (DANA). Higher frequencies of NETs are detected in subjects with RA (16).

NET formation conventionally occurs *via* the NADPH oxidase (NOX) and ROS-dependent suicidal pathway in which neutrophils rupture and release NETs. Suicidal NETosis is triggered by the engagement of specific receptors or other biomolecules, such as IgG-Fc receptors, Toll-like receptors (TLRs), complement molecules, and cytokines on neutrophils (11, 17–19). The formation of suicidal NETosis is a gradual process that is commonly initiated by the generation of ROS. Then NE and MPO are transported into the nucleus where histones are modified. Finally, nuclear and cellular membranes break, and NETs are released (9, 10, 14). During this process, substantial morphological changes occur. Neutrophils flatten and form membrane protrusions after stimulation (11). Nuclear lobules disappear and chromatin decondense. The inner and outer nuclear membranes detach. The nuclear membrane disintegrates into vesicles, and nuclear material mixes with the cytoplasm to form a homogenous mass. Finally, the neutrophils round up and rupture to release the NETs (10). ROS are pivotal for suicidal NETosis formation (20). ROS are mainly generated by NOX during the "respiratory burst" of neutrophils (21). Patients with chronic granulomatous disease harbor mutations of NOX genes and show reduced NET formation (10, 22). Finally, the NOX complex converts molecular oxygen to hydrogen peroxide, which is a substrate of MPO and is sufficient to induce NET formation (10). The reaction of hydrogen peroxide and MPO can form hypochlorous acid. The latter induces the generation of chlorinated polyamines that may cross-link NET proteins, which maintains the ordered structure of NETs and increases the capacity to trap bacteria (23). MPO partly binds to NE to form the azurosome complex that spans granular membranes without dissolution of the granular membranes (24). NE is a critical enzyme involved in many pathways of NET formation. Methicillin-resistant *Staphylococcus aureus* (MRSA) infected mice with NE deficiency fail to form NETs (25). However, other studies have demonstrated that NE is not required for NET formation induced by noninfectious stimuli; meanwhile, NE deficiency has little effect on histone citrullination (26). NE combines with F-actin filaments to enter the nucleus before MPO. The proteolytic activity of NE is determined by MPO. Patients with mutant MPO also show reduced NET formation (22). As NE translocates from cytoplasm to nucleus, it cleaves histones and participates in chromatin decondensation (27).

In addition to chromatin decondensation performed by NE, another important chromatin modification is histone citrullination driven by PAD4. During NET formation, calcium influx of neutrophils activates a high amount of PAD4, which catalyze histone to citrullinated protein. This enzyme citrullinates arginine residues in the core histones H2A, H3, H4, thus reducing their positive charge, which weakens the interactions between histones and DNA and further promotes chromatin decondensation (28). Five calcium molecules are bound to every PAD4 molecule (29). Interestingly, citrullination driven by PAD4 is inhibited accompanied by inhibition of NOX (28), which may be due to the NOX-induced increase in cytoplasmic calcium levels that activate PAD4 (30). Citrullination driven by PAD4 is induced by LPS and PMA (31). PADI4-deficient mice failed to form NETs after treatment with certain stimuli (32–34).

However, Clark et al. first reported vital NETosis in which neutrophils remained impermeable for SYTOX Green after releasing NETs, which suggested that the neutrophils maintained an intact plasma membrane during NET formation (7). Subsequent researchers found that NETs were induced by blebbing of the nuclear envelope and vesicular exportation in *S. aureus* infection (35). Vital NETosis is activated by pathogen-associated molecular patterns (PAMPs) or endogenous damage-associated molecular patterns (DAMPs). Stimuli recognized by TLR4, such as LPS, may initiate vital NETosis. The nucleus loses its multilobular shape and becomes rounded. Then, the nuclear double membranes vanish, and vesicles composed of DNA filaments bud. These vesicles approach toward the plasma membrane. DNA is released through a small area on the cell surface. Suicidal NETosis and vital NETosis can be distinguished by cleaved N-terminal histone tails (36).

ROLES OF NETS IN RA

Pathogenesis of RA Related to NETs Formation

Various elements in the peripheral blood of patients with RA, such as autoantibodies or immunostimulatory molecules, reportedly stimulate NET formation (**Table 1**). Excessive NET formation leads to the production of deaminated antigens such as citH2A, citH2B, and citH4 histones (**Figure 1**). Furthermore, NET-borne citrullinated vimentin is a pivotal autoantigen that stimulates the secretion of proinflammatory cytokines (e.g., TNF-α and IL-1) and the expression of PADI4 and receptor activator of nuclear factor kappa B ligand (RANKL) in fibroblast-

TABLE 1 | Stimuli inducing neutrophil extracellular traps (NETs) formation.

Pathogen triggers	Endogenous triggers	Inflammatory triggers
Bacteria	TLR 4	Antibodies (40)
Viruses	FcγRIIIb (37)	Immune complexes (19)
Fungi	IL-8, IL-17, TNF-α (38)	Lipophosphoglycans (41)
Protozoa	IFN-γ (39)	M1 protein (42)
	Calcium salt crystals	LPS (8)
	Urate crystals	Hydrogen peroxide (10)
		PMA (6)
		Calcium ionophore A23187 (43)

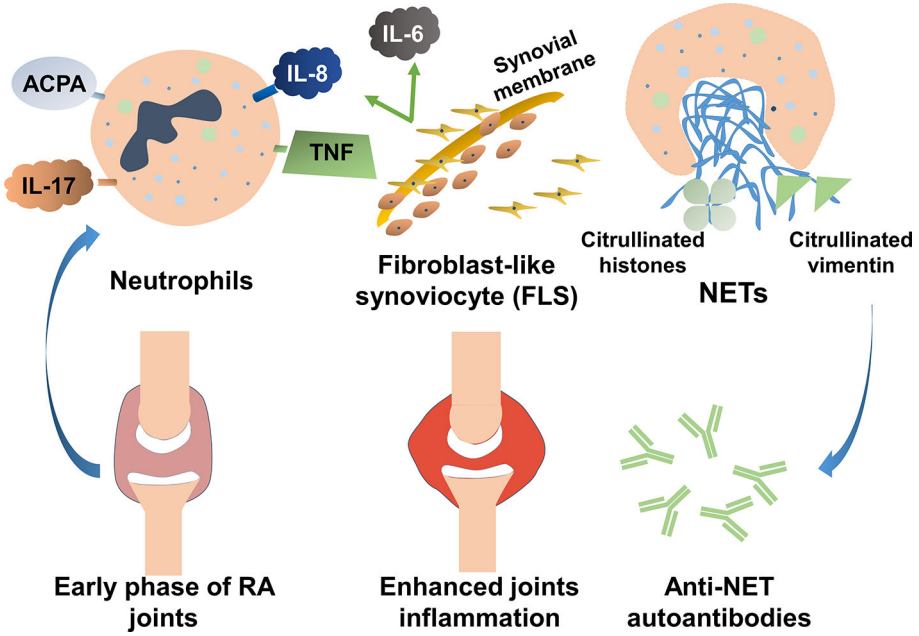

FIGURE 1 | The role of neutrophil extracellular traps (NETs) in the pathogenesis of rheumatoid arthritis (RA). Various elements in the peripheral blood of patients with RA can stimulate NET formation. Excessive NET formation leads to the production of deaminated antigens such as citH2A, citH2B, and citH4 histones. In a joint with RA, presented citrullinated antigens induce antigen-driven autoimmune responses and lead to the generation of anti-NET autoantibodies. Thus, persistent inflammation of the synovial membranes occurs.

like synoviocytes (FLSs) (44). FLSs, key effector cells of inflammation in RA, produce multiple cytokines that cause damage in the joints (45). In a joint with RA, presented citrullinated antigens induce antigen-driven autoimmune responses and lead to the generation of anti-NET autoantibodies. Thus, chronic inflammation and autoimmunity exist for a long time. Anti-NET RA recombinant monoclonal antibodies (rmAbs) derived from CD19+ synovial B cells of patients with RA constantly bind NETs.

The immunoreactivity of NET-Ags depends on somatic hypermutation (SHM) within the Ig variable H (VH) and variable L (VL) chains of synovial B cells. Moreover, Fab-N-linked-glycosylation determines the reactivity of the autoantibodies (46). Rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPAs), and other autoantibodies in peripheral blood or synovial fluid robustly support NET formation in RA (47). IgG or IgM collected from peripheral blood or synovial fluid of patients with RA induce more NET formation than antibodies from healthy controls (38). Recently, NET-derived elastase results in cartilage matrix disruption and induction of membrane-bound peptidylarginine deiminase-2 released by FLSs. Cartilage fragments are subsequently citrullinated and presented to antigen-specific CD4+ T cells (48). In the NOX/ROS pathway, PAD4-induced histone citrullination promotes chromatin decondensation and NET formation (49). The chromatin-associated protein DEK regulates the structure of extracellular chromatin (50, 51). In models of RA (51), NET formation and protein citrullination are shown to be prevented by depletion of DEK or administration of DEK-targeted aptamers. Both strategies alleviate the symptoms of RA.

In patients with RA, IL-8, IL-17A, and TNF-α reportedly induced NET formation (38). Upon exposure to IL-17A, neutrophils in RAlead to NET formation when the cells are primed with TNF-α. Correlations with NET formation have also been detected for a higher serum level of C-reactive protein (CRP) and a higher erythrocyte sedimentation rate. Furthermore, histone citrullination alone with NET formation can be triggered by the treatment of neutrophils with supernatants harvested from IL-15-stimulated CD69(+)CD8(+) T cells, leading to the extracellular release of citrullinated proteins (52). Conversely, immune complexes induce “incomplete” NET formation (53, 54). Ribonucleoprotein-containing immune complexes induce NET formation depending on mitochondrial ROS rather than NOX (55, 56), which correlates with hypercitrullinated proteins (57) and production of IFN by plasmacytoid dendritic cells (58).

Signal Transduction Pathways Correlating With NETs

Several underlying signal transduction pathways may promote NET formation in RA and the molecular mechanisms may be pleomorphic. In RA, high concentrations of NE, MPO, PAD4/DNA-complex, and ROS production correspond to the elevated formation of NETs (31, 59).

In neutrophils, Rac is a subunit of the NOX complex (60). Guanosine exchange factor (GEF) activator, Vav, and the p21-activated kinases (Paks) are involved in Rac signaling pathway (61). NOX is indispensable for oxidative burst-dependent NET formation (60–62). Inhibition of NOX reduces NET formation and induces non-canonical NETs (10). ROS are related to the lytic NET formation (63) and stimulate the activation of NE. NE and MPO are also released from azurophilic granules into the nucleoplasm (25). In the nucleus, NE proteolytically cleaves histones and thus interferes with the dense package of chromatin (27). Many physiological and artificial stimuli can activate the MPO-NE pathway (22).

In the peripheral blood of patients with RA, ACPAs stimulate neutrophils to release PAD enzymes (64). *Porphyromonas gingivalis* and smoking are known risk factors for RA, and overexpression of endogenous or bacterial PAD enzymes drives citrullination (65, 66). PAD4 depends on Ca^{2+} (67) and is activated *via* the ROS pathways to convert internal arginine to citrullines (8, 9). Upon PAD4 activation, locally released citrullinated histones enhance the generation of highly mutated clonal B cells resulting in the generation of high-affinity ACPAs (68). At a high titer, fibrinogen citrullinated by PAD 4 acts as the preferred targets for ACPAs (69). Additionally, human leukocyte antigen (HLA)-DR bound PAD4 is recognized by T cells and further contributes to the production of antibodies responded to citrullinated proteins, such as ACPAs and anti-PAD antibodies (70). Anti-PAD4 antibodies have been reported to be closely related to ACPAs (71–73). Kolfenbach et al. evaluated the prediagnosis serum samples of 83 patients with RA and found that 15 RA samples had anti-PAD4 antibodies with a high specificity of 98.8% (71). Interestingly, Erika Darrah et al. first detected PAD4-specific CD4+ T cells in peripheral blood mononuclear cells (PBMCs) of RA patients and found that protease granzyme B (GrB) induced structure changes of PAD4 and promoted the presentation of CD4+ T cell epitopes (74). Overall, further studies are need to demonstrate correlation between PAD4 and RA citrullinome.

Deficiency or inhibition of PADI4 reduces the formation and the size of NETs and alleviates arthritis symptoms in many models, except the murine K/BxN model (75). These findings suggest that PADI4 acts downstream of ROS in NET formation and generates autoantigens that amplify the inflammatory response that precipitates in the pathogenesis of RA (32, 76, 77). PADI4 thus participates in the initiation rather than the effector phase of RA. PAD4, which is associated with histone deamination, can catalyze hypercitrullination by immune-mediated membranolytic pathways (57, 78). Interestingly, in a TNF-induced model of citrullination and arthritis, protein citrullination is executed by PAD2 instead of PAD4. PAD2 is not associated with NET formation (79). Relatively high activity of putatively neutrophil-derived PAD4 has been reported in RA synovial fluid (80). The pathogenesis of RA is also related to T cells specific for citrullinated epitope (81). In brief, the NOX pathways and PAD4 activity can be regarded as critical elements that regulate NET formation and generation of citrullinated autoantigens in RA (82).

NETs Promote Autoantibody Production, Cytokine Expression, and Cell Activation

Citrullinated components of NETs often serve as self-antigens recognized in the serum of patients with RA (80). Aberrant NETs may promote the externalization of citrullinated autoantigens and immunostimulatory molecules, which enhances the expression of epitopes related to the pathogenesis of RA (38). In RA or osteoarthritis (OA), the levels of IL-6 and IL-8 are upregulated in the presence of NETs, resulting in the activation of FLSs (38, 83). LL-37/DNA complexes induce NETs that activate plasmacytoid dendritic cells *via* TLR7 and TLR9 to produce type I IFN (84). Moreover, NETs are abnormally accumulated in some patients with SLE due to the DNase I inhibitory factors (85, 86), leading to IFN-a release. IFN-a not only enhances NETosis but also induces activation of autoreactive T- and B cells to synthesize autoAbs, such as anti-dsDNA, anti-HNP, and anti-LL37 autoAbs. Moreover, NETs can trigger the production of IL-1β and IL-18, and further stimulate NETosis. These vicious cycles contribute to the imbalance immune homeostasis of SLE. Similarly, NETs are involved in multiple sclerosis (MS). NETosis secretion of antimicrobial proteins induces elevated T-cell activation resulting in tissue damage in MS (87).

NETs triggered by microscopic cholesterol crystals also take part in atherosclerosis (88). NETs induce the activation of leukocytes, platelets, and endothelial cells and further lead to endothelial dysfunction (89). Moreover, NETs promote the production of IL-6 and pro-IL-1β in macrophages (49). Accordingly, these increased cytokines accelerate T helper 17 (TH17) cells differentiation and subsequently induce immune cell recruitment in atherosclerotic lesions. Neutrophils infiltration of culprit lesions results in plaque rupture and erosion *via* NETs (90, 91). Very recently, significantly higher plasma levels of NETs are observed in the carotid lesion site (CLS) of stroke patients. NETs decorated with phosphatidylserine (PS) are detected in thrombi. NET formation requires the synergy of CLS plasma and activated platelets (PLTs). PS-bearing NETs can induce the formation of thrombin and fibrin as well as the conversion of endothelial cells to a procoagulant phenotype (92). These findings indicate that NETs are indispensable in the pathogenesis of many diseases, such as RA, SLE, MS, atherosclerosis, and stroke *via* multiple molecular mechanisms.

NET and Citrullinated Autoantigens Form a Vicious Cycle in RA

In RA, neutrophils infiltrate synovial tissue, rheumatoid nodules, and the skin (38), when neutrophils form NETs, deaminate proteins, and initiate ACPA production (57). Furthermore, circulating low-density granulocytes (LDGs) in patients with RA tend to increasingly form NETs (19). FLSs activated by NETs express IL-17A, TNF-α, and IL-8 and infiltrate the cartilage, where they enhanced proinflammatory responses (10). The enhanced release of inflammatory cytokines from FLSs driven by NETs causes joint damage and further worsens the condition (31). The secretory leukocyte protease inhibitors can prevent proteolytic maturation of cytokines related to NET formation. Skin lesions may be associated with the insufficient

activity of the secretory leukocyte protease inhibitors (93). Importantly, these cytokines trigger the vicious cycle of NET formation and autoantibody biogenesis (38). IL-8 and IL-17 recruit neutrophils and promote the exposure of autoantigens (12, 94). Therefore, NET formation plays a critical role in the pathogenesis of RA. A comprehensive understanding of the mechanisms involved in NET formation may help us develop new therapies *via* targeting NETs to treat NET-related diseases.

NETS ARE ASSOCIATED WITH RA IN MOUSE MODELS

Neutrophils Drive the Inflammation of Murine Arthritis

RA is an autoimmune disease characterized by progressive destruction of joints. The pathogenesis of RA is still elusive. Researchers usually establish murine models to analyze the pathogenetic sequelae of RA (95). Daisuke and colleagues established an experimental model of male BALB/cAnNCrj (BALB/c) mice injected with an anti-type II collagen antibody and LPS (95). Histological analysis showed that neutrophils were the vast majority of infiltrating cells in the joint space. To determine the effect of neutrophils on arthritis, monoclonal antibodies (mAbs) against Gr-1 (the RB6-8C5 mAb) were intravenously injected into arthritic mice to deplete circulating neutrophils. These experiments suggested that neutrophils are indispensable for the development of arthritis. It is commonly believed that neutrophils play a key role in inflammatory diseases due to their secretion of cytotoxic products (4). However, neutrophils are now considered to be not only effectors of the innate immune systems but also key players in the regulatory circuits of the immune system (96). FcγRs activate neutrophils and trigger a series of signaling events, including ROS generation, protease release as well as the production of chemokines and cytokines. These mediators recruit additional neutrophils and regulate the functions of other immune cells. Hence, they participate in the regulatory network and interplay of innate and adaptive immunity (97). Neutrophils isolated from patients with RA functionally differ from those from healthy controls. Blood- and synovial fluid-derived neutrophils from patients with RA trigger ROS production and display enhanced NET formation (38).

The Role of NETs in the Etiopathogenesis of RA

NETs are considered to contribute to the pathogenesis of RA (9). Degradation or citrullination of histones driven by PAD4 promotes chromatin decondensation and NET release (38). Furthermore, PAD4 exacerbates inflammatory arthritis and is crucial in some pathways of NET formation (79). Compared to wild-type mice, PADI4 (encoding PAD4)-deficient mice induced by glucose-6-phosphate isomerase showed less severe inflammatory arthritis and reduced autoantibody titers (75). Similarly, in murine collagen-induced arthritis (CIA), inhibition of PADI4 reduced the formation of NETs and arthritis relief (98). However, PAD4 was dispensable in spontaneous arthritis in the K/BxN mouse model (99). NET formation and arthritis in the murine TNFα-induced inflammatory arthritis were investigated to identify the roles of PAD2 and PAD4 for citrullination. PAD2 mediated TNFα-induced citrullination and arthritis but was dispensable for NET formation (99). PAD4, which is involved in NET formation, was dispensable for citrullination. These studies indicate that various pathogenic pathways may cause murine arthritis. These can be dependent or independent of NETs.

Further evidence supported the roles of NETs in the pathogenesis of RA that blocked NET formation and protein citrullination was caused by treatment with DEK-targeted aptamers, as DEK is essential for certain pathways of NET formation (38, 100). Autoantibodies that recognize DEK have been detected in the sera of patients with autoimmune diseases, such as systemic lupus erythematosus (SLE) and adolescent idiopathic arthritis (JIA) (100). DEK acts as a chemoattractant, triggers inflammatory responses, and plays an important role in a murine model of arthritis. Aptamers targeting DEK could reduce NETs formation, slow the progression of joint inflammation, and ameliorate the disease symptoms in arthritic mice (51).

Another established murine model of RA is the K/BxN mice. The pathology is similar to that of human RA. The K/BxN mice are generated by crossing KRN-C57BL/6 mice, which carry a transgenic T cell receptor, with autoimmunity-prone non-obese diabetes (NOD) mice (101). K/BxN mice develop IgG autoantibodies against glucose-6-phosphate isomerase, which precipitate joint damage.

Mice lacking functional NOX have the further aggravation of arthritic symptoms. ROS suppression occurs in patients with chronic granulomas disease (CGD) due to impaired function of NOX (35). This implies that ROS in NETs are derived from additional mechanisms beyond the NOX pathway (86). NET formation triggered by nicotine (102) was found to be dependent on mitochondrial ROS rather than depend on NOX (20). However, Cl-amidine, a PAD inhibitor, did not inhibit the formation of mitochondrial ROS but inhibit NETs in the New Zealand mixed 2328 (NZM) murine mice (34).

DIAGNOSING RA WITH NETS

Currently, the laboratory diagnosis of RA relies on the detection of RF and ACPAs (59, 103). These autoantibodies can be found in most RA patients, and the titer of ACPAs correlates with the severity of RA (4). Although many autoantibodies markers have been applied for patients' diagnosis with RA, ACPAs are the most disease-specific markers with the highest specificity and sensitivity (103). Khandpur et al (38). analyzed 55 Patients with RA and 36 healthy volunteers or patients with OA. The results showed that NET formation was associated with the levels of ACPAs and indicated NETs were a potential target for ACPAs. ACPAs include antibodies targeting keratin (AKA), perinuclear

factor (APF), profillagrin/fillagrin (AFA), Sa, and artificial cyclic citrulline peptide (CCP). The diagnostic specificity of four kinds of ACPAs (APF, AKA, AFA, and CCP (II)) for RA was more than 90%, which is significantly higher than that of RF (77.7%), suggesting that ACPAs can be employed as effective diagnostic antibodies for RA. However, the sensitivity of ACPAs for RA differs due to differences in antigens preparation and detection methods (104–106).

Recently, some studies have focused on the detection of potential signaling pathways that lead to the increase of NET formation in RA. This is to determine whether the products of NET formation are useful for diagnosis. NETs as target biomarkers have been reported in many autoimmune diseases. Levels of human neutrophil peptide 1–3 (HNP 1–3), a part of NETs, were found to be significantly higher in patients with lupus nephritis (LN) than in healthy controls. They are an independent indicator of LN [P = 0.006, odds ratio (OR) = 7.5, 95% confidence interval (CI), 1.782–31.842]. Moreover, the NET-inducing capacity might be a novel biomarker of ANCA-associated vasculitis (AAV). The levels of NET degradation products, such as circulating free DNA (cfDNA), free nucleosomes, NE-DNA, and MPO-DNA complexes, are reportedly increased in patients with RA (3). NET-derived products were previously analyzed *in vitro* by microscopy and enzyme-linked immunosorbent assay (ELISA). Receiver operating characteristic (ROC) curves showed spontaneously increased formation of NETs and production of ROS in patients with RA (59). NET-derived cell-free nucleosomes in RA serum showed diagnostic value with an area under the ROC > 97% with 91% sensitivity and 92% specificity (59). No significant differences were detected between ACPA-positive and ACPA-negative patients (59). Recently, the level of anti-NET antibodies (ANETA) in RA serum was reported to be significantly higher in rheumatoid factor-positive than that in seronegative patients (107). The collective evidence suggests that the quantitative detection of the NET-derived products may be a useful complementary tool to identify individuals at risk and to monitor patients with RA.

THERAPEUTIC TARGETING OF NET FORMATION TO TREAT RA

Clearance of NETs

DNAse-1 dismantles NETs *in vitro*. *In vivo*, DNase-1 does not interfere with NET production, but fragments the DNA and destroys the backbone of the NETs (9). DNase-1 reportedly promotes the escape of group A *Streptococcus* (GAS) from being killed by NETs. Enhanced neutrophil depletion of GAS and reduced virulence occur in the presence of the DNase I inhibitor G-actin (108). Recently, several studies have reported that bacterial DNases degrade NETs, allowing the bacteria to escape killing in NETs (109–111). However, Bryan et al. injected *S. aureus* intraperitoneally into mice and monitored the infections with minimally invasive nonsurgical luminescent imaging, showing that DNase reduced bacterial growth (112). Kolaczkowska and colleagues also demonstrated that DNase effectively eliminated NET-borne DNA and inhibited the proteolytic activity of NE (25). Nevertheless, NETs still show some antimicrobial activities, as most of the histones remained. The circulating zymogen form of factor VII activating protease (FSAP) can be activated by histones and the nuclear lobules of NETs. NETs bound to FSAP fail to activate pro-FASP. However, histones release after the degradation of NETs by DNase dramatically stimulating pro-FASP activity (113). Pathogenic micro-organisms produce DNases that inhibit the generation of ROS in the later stage and lead to escape the killing in NETs (114). In addition, NETs are cleared *via* phagocytosis of macrophages, which increases the release of cfDNA (115). Whether the pathogenesis of RA involves macrophage dysfunction needs further examination. The cfDNA levels in synovial fluids were correlated with neutrophil counts but not with macrophage counts (80). The increased NETs levels in RA synovial fluids may be caused by either impaired activity of DNase-1 or by inhibitors of DNase-1. Serum DNase-1 activity is negatively correlated with inflammatory markers and neutrophil counts, suggesting that insufficient DNase-1 activity is an important factor in the regulation of NET persistence. The elevated cfDNA levels in the synovial fluid may be an important source of "altered self." Only very few eosinophils and mast cells exist in the synovial fluid of patients with RA, suggesting that cfDNA are mainly derived from NETs. An advantage of DNase-1 is low toxicity, which has been verified in murine models of breast cancer (116), lupus (117), or lung damage (118). Exogenous administration of DNase I may be used to dismantle NETs and can, therefore, be considered for the treatment of RA.

Inhibition of NET Formation

Additionally, drugs that reduce the formation of NETs may also be used to treat and relieve RA. Delivery of the NE inhibitor sivelestat *via* a nanoparticle system to LPS-induced endotoxin shock mouse model inhibits NET formation, reduces circulating NE, and prevents mice from endotoxic shock (119). CI-amidine can reduce protein citrullination in the pGIA mouse model (120). Rituximab and belimumab reduce NET formation by blocking the immune-complexes formation (121). Emodin accelerates apoptosis and suppresses autophagy and NET formation by reducing IL-6, IFN-γ, and TNF-α in the murine adjuvant-induced arthritis (AA) (122). Polydatin (PD) reduces NET formation of bone marrow-derived neutrophils and in patients with RA. Similarly, in CIA mice, the deposition of NETs in the ankle joints is decreased by PD-treatment (123). Ascomycin and cyclosporine A can decrease IL-8 induced NET formation by inhibiting the calcineurin pathway (124). Triptolide (TP) exhibits potential as an RA therapeutic by lowering neutrophil recruitment and downregulating the expression of TNF-α and IL-6. TP is also able to suppress NET formation and autophagy of neutrophils (125). Moreover, celastrol can inhibit NOX-dependent NET formation (126). Tocilizumab likewise shows the potential to reduce autoantibody levels and, consequently, immune complex formation in patients with RA (127). Nevertheless, there are

differences in the immune system and physiological function between murine models and humans. Given the limited scope of current clinical standard therapies, more clinical research is required to establish NET reducing therapies.

PERSPECTIVES AND CONCLUSION

The accumulated data on the role of NETs in RA has brought NETs into focus as novel therapeutic targets for RA. The future will tell whether a blockage in NET formation or increased NET catabolism will win the race.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Extracellular Vesicles Secreted by Mesenchymal Stromal Cells Exert Opposite Effects to Their Cells of Origin in Murine Sodium Dextran Sulfate-Induced Colitis

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Several reports have described a beneficial effect of Mesenchymal Stromal Cells (MSCs) and of their secreted extracellular vesicles (EVs) in mice with experimental colitis. However, the effects of the two treatments have not been thoroughly compared in this model. Here, we compared the effects of MSCs and of MSC-EV administration in mice with colitis induced by dextran sulfate sodium (DSS). Since cytokine conditioning was reported to enhance the immune modulatory activity of MSCs, the cells were kept either under standard culture conditions (naïve, nMSCs) or primed with a cocktail of pro-inflammatory cytokines, including IL1β, IL6 and TNFα (induced, iMSCs). In our experimental conditions, nMSCs and iMSCs administration resulted in both clinical and histological worsening and was associated with pro-inflammatory polarization of intestinal macrophages. However, mice treated with iEVs showed clinico-pathological improvement, decreased intestinal fibrosis and angiogenesis and a striking increase in intestinal expression of Mucin 5ac, suggesting improved epithelial function. Moreover, treatment with iEVs resulted in the polarization of intestinal macrophages towards and anti-inflammatory phenotype and in an increased Treg/Teff ratio at the level of the intestinal lymph node. Collectively, these data confirm that MSCs can behave either as anti- or as pro-inflammatory agents depending on the host environment. In contrast, EVs showed a beneficial effect,

suggesting a more predictable behavior, a safer therapeutic profile and a higher therapeutic efficacy with respect to their cells of origin.

Keywords: inflammatory bowel disease, mesenchymal stromal cells, extracellular vesicles, macrophage polarization, sodium dextran sulfate, immunomodulation

INTRODUCTION

Inflammatory Bowel Diseases (IBD) such as Ulcerative Colitis and Crohn's Disease are chronic inflammatory diseases of the gastrointestinal tract. Although the origin of IBD remains obscure, the widely believed hypothesis is that environmental factors or infections can alter the barrier function of the epithelium, leading to loss of immune tolerance to intestinal antigens (1, 2). The immune response in the intestine is a tightly controlled balance between innate and adaptive effector responses and negative regulatory pathways. Dysregulation of the immune response and alteration of the equilibrium between protective immunity and tolerance to self-antigens and commensal bacteria have been emphasized as pathogenic factors (1, 2). Drugs used to suppress the immune response and reduce tissue inflammation are not always effective and are often associated with more or less serious side effects (3). Mesenchymal stromal cells (MSCs) have shown beneficial effects in animal models of IBD and have been approved by the European Medicines Agency for the treatment of fistulas in Crohn's disease following a successful phase II/III clinical trial (4). In their clinical application for the treatment of immune disorders, however, administrated MSCs might encounter a biased cytokine milieu *in vivo*, which could actually render MSCs immune-enhancing (5). Indeed, in some conditions MSCs could promote the proliferation of suboptimally activated T cells (6, 7).Therefore, MSCs appear to respond to environmental signals possibly resulting in unpredictable opposite behavior *in vivo*.

It is now well established that several beneficial effects of MSC transplantation are mediated by paracrine signaling (8–10), and that such signals are mostly convoyed *via* membrane vesicles released by the cells, named extracellular vesicles (EVs) (11–13). EVs are a heterogeneous population of nanoparticles up to 1 μm in size, including exosomes and microvesicles, carrying both effector molecules and RNAs (14). The discovery that EVs secreted by MSCs can reproduce some immunomodulatory and pro-regenerative effects of their cells of origin has prompted investigations on the use of these cell products as therapeutic tools, and indeed their beneficial effects have been confirmed in several animal models of organ and tissue injury (15). In particular they were effective in improving both clinical and histological signs of colitis in animal models (15). We also observed similar therapeutic effects of MSCs and EVs in hyperoxia-induced lung injury (16).

Following the recognition of the therapeutic potential of exosomes, several researchers are trying to modify their composition to enhance specific biological effects on target cells. Several authors have shown that the immunomodulatory activity of MSCs is affected by cytokine priming, both *in vitro* and *in vivo* (17–20). However, only few studies have investigated the effects of MSC priming by pro-inflammatory cytokines on the biological activity of released EVs (21, 22). Here, we investigated the effects of both MSCs and EVs, with and without priming with pro-inflammatory cytokines, in a well-established murine model of IBD. Surprisingly, we observed a divergent effect of MSC-EVs vs. their parent cells, a difference that was amplified by cytokine conditioning.

MATERIALS AND METHODS

Murine MSC Isolation and Culture

Murine MSCs were isolated by flushing the bone marrow of C57BL6/J mice. Cells were cultured in 25 cm^2 tissue culture flasks at a concentration of 2.00E+6 cells/cm^2. MSCs were fed in Dulbecco's modified eagle medium low glucose [1g/L] (DMEM; Gibco by Life Technologies) enriched with 10% fetal bovine serum (FBS; Gibco by Life Technologies), [100 U/mL] penicillin and streptomycin (P/S; Gibco by Life Technologies), [100 mg/mL] L-glutamine (Gibco by Life Technologies) and incubated at 37°C, 5% CO2. After 48 hours, the non-adherent cells were removed. After reaching 80% confluence, the adherent cells were trypsinized (0.05% trypsin at 37°C for 3 minutes), harvested and expanded in larger flasks. MSC at passage 5 were screened by flow cytometry (LSRFortessa (BDBiosciences) for the expression of SCA-1, CD31 and CD34 (BD Pharmingen, Oxford, UK) and used to perform the experiments. MSCs were grown up till reach 80% of confluency in ventilated cap flask. Growth medium was substituted with DMEM low glucose [1g/L] supplemented with 10% FBS, 2mM glutamine, 100 U/ml penicillin/streptomycin, without (for naïve MSCs) or with a pro-inflammatory cytokine cocktail (for induced MSCs: iMSC) composed by IL-6 [20 ng/mL], TNF-α [25 ng/mL] and IL-1β [25 ng/mL] (Peptrotech) for 24 hours. This medium was changed with DMEM low glucose supplemented with 2mM glutamine, 100 U/ml penicillin/streptomycin for 24 hours before collection for EV isolation (20, 23).

Extracellular Vesicles by Ultrafiltration

Culture medium (CM) was centrifuged at 1200 rpm for 6 minutes to discard dead cells and debris and filtered through 0.22 μm filter (filter unit syringe driven, Millex-GP). Supernatant was loaded onto an Amicon filter device (Amicon filters Ultra-15, regenerate cellulose 100,000 NMWL; Merck Millipore). The filter unit was centrifuged at 3200g at 4°C for 15 minutes and the concentrate was collected.

Measurement of Extracellular Vesicles Concentration and Size Distribution

Particle concentration and size distribution were analyzed by Tunable Resistive Pulse Sensing (TRPS) technology with the qNano instrument (Izon Science, Christchurch, New Zealand).

In this system, a membrane including a tunable submicron-sized pore separates two fluid chambers, one containing the sample to be analyzed, the other an electrolyte solution. A voltage is applied across a membrane, resulting in an ionic current. While passing through the pore, the particles generate a "blockade" event, which in turn generates a pulse that is directly proportional to the particles' volume, while the blockade rate is related to particle concentration. In the present setup, a NP150 membrane was used. The concentration of particles was standardized using a CPC100 calibration solution diluted 1:10000 (110 nm mean carboxylate polystyrene beads; raw concentration 1.00E+12).

Functional Assays

Macrophage Functional Assay

RAW 264.7 murine macrophages (passages 12-15) were cultured in DMEM High Glucose 5% FBS. For the assay, cells were seeded in sextuplicate in a 96-well plate (40.000cel/well) for 24h and were stimulated with or without LPS from E. coli O111:B4 (L4391, Sigma) at a dose of 10ng/mL, plus dexamethasone (D4902, Sigma) at a dose of 1μg/mL or plus nEV or iEV (5,00E+7, 5,00E+8, 5,00E+9 EV) for 16h. After that, cell culture supernatant was taken and analyzed for IL-10 concentration following manufacturer's instructions (mouse IL-10 ELISA kit DY417, R&D).

Endothelial Functional Assay

80 μl of Matrigel Matrix (Corning) were seeded in the 96-well plate and left to polymerize at 37°C, 5% CO2 for at least 30 minutes. SVEC4-10 cells with 5,00E+9 EVs were resuspended in 96μl DMEM, supplemented with 10% FBS seeded on the solidified matrix and incubated for 4 hours at 37°C 10% CO2. DMEM low glucose with 10% heat-inactivated FBS was used as control. After the incubation, pictures were taken with a phase contrast inverted microscope (Olympus) and analysis were performed with ImageJ Angiogenesis Analyzer.

Western Blot Analysis

For the western blot analysis, both naïve and induced mMSC and EV were lysed in RIPA buffer and then incubated with Laemmli buffer with β-mercaptoethanol for 5 min at 95°C, for complete protein denaturation. Then, samples were loaded and resolved in an SDS-polyacrylamide 4-12% gel at 140V and blotted using semi-dry transfer for 7 min at 25V to polyvinylidene difluoride membranes (PVDF) (GE Healthcare Life science). Membranes were blocked with 5% BSA in TBS-Tween for 1h at room temperature and then incubated with primary antibodies overnight: mouse monoclonal ALIX antibody 1:500 (MA183977, Thermofisher), rabbit polyclonal TSG-101 antibody 1:1000 (ab-125011, Abcam), diluted in 1% BSA in TBS-Tween. After washing, membranes were incubated with secondary antibodies: goat- anti rabbit-HRP 1:5000 (65-6120, Thermofisher) and goat anti-mouse-HRP 1:5000 (62-6520, Thermofisher) for 1h at room temperature. After washing, bands were evidenced by means of ECL Plus Western blotting analysis system (32134, Thermofisher).

Transmission Electron Microscopy (TEM)

One drop of EVs solution (about 25μl) was placed on 400 mesh holey film grid; after staining with 1% uranyl acetate (for 2 minutes) the sample was observed with a Tecnai G2 (FEI) transmission electron microscope operating at 100 kV. Images were captured with a Veleta (Olympus Soft Imaging System) digital camera.

Mouse Model of Colitis

C57BL/6J mice were purchased from Charles River Laboratories (Calco, Italy). All mice used as primary cell donors or recipients were between 8 and 12 weeks of age. Procedures involving animals and their care conformed to institutional guidelines in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international (EEC Council Directive 2010/63/UE; National Institutes of Health Guide for the Care and Use of Laboratory Animals) law and policies. The protocol was approved by the Italian Ministry of Health, Division of Veterinary Medicine (protocol n°861/2016-pr, risp. a prot c35de.2 #195042387#). All efforts were made to minimize the number of animals used and their suffering. Colitis was induced in C57BL/6N mice by administration of 3% dextran sulfate sodium (DSS) (molecular mass, 40 kDa; Sigma Aldrich) in drinking water for 6 days followed by 3 days on plain water. Mice with colitis were divided into five treatment groups as described in **Table 1** and received an intraperitoneal injection (IP) of MSCs on days 4 and 8 or of EVs on days 4, 6 and 8. Both MSCs and EVs were suspended in 200 μL of PBS. Control mice received PBS only. To administer an amount of EVs approximately proportional to the number of injected MSCs, we calculated the EV dose based on the following assumptions: i) the transplanted MSCs remain active in the host tissue for about 24-48 hours (24, 25); ii) the transplanted MSCs produce an amount of EVs comparable to that produced *in vitro* in standard 2D culture conditions in this time period (about 1.00E+3 EVs per cell according to our experience). The severity of colitis was assessed daily by measurement of weight loss and of disease activity index (DAI). DAI was calculated on the degree of diarrhea and of visible fecal blood as described by Cooper et al. (26). Mice were euthanized on day 10. The entire colon was removed, and colon length was measured. The colon was opened longitudinally and rinsed with physiological saline to remove fecal residues. Tissue samples were stored in liquid nitrogen for subsequent analysis or embedded in paraffin.

TABLE 1 | Experimental groups.

Group	N	Intraperitoneal (IP) Treatment
Healthy, untreated (UT)	5	none
Colitis + vehicle only (PBS)	5	200μL/administration
Colitis + MSCs	5	4.00E+6/administration
Colitis + iMSCs	5	4.00E+6/administration
Colitis + nEVs	5	1.00E+9/administration
Colitis + iEVs	5	1.00E+9/administration

Histopathological Analysis

The colon, from the rectum to the ileo-cecal junction, was entirely sampled for histopathological evaluation. It was cut longitudinally and fixed in 10% buffered formalin for 2 days. The colon was then paraffin-embedded; 5-µm-thick sections were sliced and stained with Hematoxylin and eosin. The colonic mucosa had a quite patchy aspect, showing the alternation of lesions and almost normal mucosa. The intestinal lesions were evaluated through the following score: 0, no inflammation or crypt damage; 1, mild inflammation and damage of the basal 1/3 of crypts; 2, moderate inflammation and damage of the basal 2/3 of crypts; 3, severe inflammation with total crypt loss, although in the presence of surface epithelium; 4, severe inflammation with total crypt loss, in the absence of surface epithelium. The total colitis score was then calculated by multiplying the above score for the percentage of damaged colonic mucosa. In particular, this percentage was calculated by dividing the total length of the injured colonic areas for the total length of the colon (from the rectum to the ileo-cecal junction and without considering the cecum).

Lymph Node Cell Isolation and Flow Cytometry

Single cells suspensions were prepared from mesenteric lymph nodes (MLN) by forcing the organs through an 80µm mesh (Sigma-Aldrich). Cells were washed twice with PBS and then suspended in Flow Cytometry Staining Buffer (eBioscience). To block unspecific binding of antibodies, cell suspensions were incubated with an anti-CD16/32 mAb (2.4G2, eBioscience) for 15 min on ice and then stained with combinations of the following fluorochrome-labeled antibodies against surface markers for 30 min on ice: CD3 (17A2, eBioscience), CD4 (GK1.5, eBioscience), CD69 (H1.2F3, Abcam), and CD25 (PC61.5, eBioscience). Intracellular staining for FoxP3 (FJK-16s, eBioscience) was performed using the FoxP3/ Transcription factor staining buffer set (eBioscience) according to the manufacturer's instructions. Flow cytometric analysis was performed using a FACSCalibur based on CellQuest software (BD-Becton Dickinson, Franklin Lakes, USA). T cell effectors (Teff) were defined by: CD3, CD4, CD69+. T cell regulators (Treg) were defined by: CD3, CD4, CD25, FoxP3+.

Immunohistochemistry

Slides Deparaffinization

Slides were incubated twice for 5' in xylene, and then hydrated in a series of ethanol solution at decreasing concentration (100%/100%/95%/80%) for 3 minutes each and then placed in distilled water. To completely deparaffinize samples without disrupting their morphology and without damaging surface epitopes, slices were placed in a pre-heated bath at 90°C with a Trisodium Citrate pH 6,03 buffer solution for 30 minutes. Slides were rinsed with PBS-Tween20 0.05% for two minutes.

Immunofluorescence

A solution of glycine 0,1 M to block unspecific binding with antibodies, and then permeabilized with a solution of 1% BSA and 0.02% NP40 in PBS for 1h at room temperature. Primary antibodies were diluted in 1% BSA solution in PBS and used accordingly to **Table 2**. Slides were washed in PBS and incubated with secondary antibodies at 37° C for 1 hour. Hoechst (H3570 Life technologies) 1:10000 in PBS was used for 15 minutes to mark nuclei and mounting medium (Dako S3023) was used to seal the slides. Pictures were taken using confocal LSM 800 Zeiss at 20X of magnitude. Cells positive for each antibodies and Hoechst were counted and expressed as percentage of positive cells/field.

Immunohistochemistry Was Performed With CD31

0.3% hydrogen peroxide for 10 min at room temperature was used to remove endogenous peroxidase activity and blocking serum for 30 min at room temperature. After primary antibody incubation with anti-mouse/rabbit serum (DAKO EnVision - TM Peroxidase, Rabbit, Dako Corporation, Carpinteria, CA) for 30 min at room temperature, 3,3=-diaminobenzidine (Sigma-Aldrich, Milan, Italy) was used. Counterstained with hematoxylin was performed. Negative controls were also done. Pictures were taken with a Leica DM4500B microscope coupled with a DFC320R2 camera for image acquisition and analyzed using Fiji software Color deconvolution/H DAB to analyze the signal of CD31. Threshold software was used to quantify area with positive CD31 signal, data were expressed as percentage of tissue covered per field.

Sirius Red

To quantify Collagen I on our samples, staining for Sirius red was performed. Hematoxylin staining for nuclei using Gills Hematoxylin solution nr.3 (Bio Optica 05-M06015) followed by staining with Sirius red solution (Sigma Cat #P6744-16A) following manufacturer's instruction. Mounting medium (Fluka 03989) was used. Ten random pictures were taken at 10X magnitude with Leica B5000 inverted microscope and

TABLE 2 | Antibody list.

Antibodies	Dilution	Incubation condition	Clone	Ref
CD31	1:50	O.N. 4°C	JC70A	M0823 (Dako)
Rabbit anti-iNOS	1:80	O.N. 4°C	EPR16635	Ab178945 (abcam)
Rabbit anti-CD163	1:50	O.N. 4°C	EPR19518	Ab182422 (abcam)
Mouse anti-Muc5ac	1:80	O.N. 4°C	45M1	MA512178 (Invitrogen)
Rat anti-CD45	1:100	O.N. 4°C	IBL-3/16	MCA1388 (Biorad)
Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 594	1:200	1 h 37°C	Polyclonal	A11005 (Life Technologies)
Goat anti-Rat IgG Secondary Antibody, Alexa Fluor 488	1:200	1 h 37°C	Polyclonal	A11006 (Life Technologies)
Goat anti-Rabbit IgG Secondary Antibody, Alexa Fluor 594	1:200	1 h 37°C	Polyclonal	A21442 (Life Technologies)

quantification of collagen was performed with Fiji software (plugin color deconvolution for Fast Red Fast Blu DAB), that was quantified using Fiji's Threshold software. Threshold provide us the percentage of area stained in comparison with the dimension of the picture and results were expressed as Collagen I deposition/field (%).

Quantitative Real Time pCR

RNA was extracted using Rneasy Kit (Qiagen) from samples previously snap frozen in liquid nitrogen. Quantification and assessment of RNA quality were obtained with Nanodrop 2000 (Thermoscientific). High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used and qRT-PCR was performed with Platinum® Syber Green Mix (Lifetechnologies) using Roche thermocycler. A relative quantification (RQ) was calculated by ΔΔCt methods. Beta2microglobulin was used as reference gene for normalization. Primer sequences used are listed in **Table 3**. All graphs displayed were produced with GraphPad software 6. Data are displayed as means ± standard deviation

Statistical Analysis

Normally distributed variables are presented as mean ± SD. Comparisons of categorical variables were carried out using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. $P<0.05$ was assumed statistically significant. Statistical analysis was performed by the use of IBM SPSS Statistics version 20(SPSS Inc., Chicago, IL, USA).

RESULTS

Characterization of MSCs and EVs

The naïve murine MSCs (nMSCs) and the induced MSCs (iMSCs) fulfilled the minimal criteria that define MSCs (27); they appeared spindle shaped and distributed as swirling (**Supplementary Figures 1A–C**). Moreover, both nMSCs and iMSCs were positive for the classical mesenchymal markers Sca1-A, CD105, CD44, CD29 and negative for the hematopoietic markers CD45, CD117 as demonstrated by the flow cytometric analysis (**Supplementary Figures 1B, D**). EVs expressed CD9, CD63 (23) as well as ALIX and TSG101 (**Figure 1F**). Tunable Resistive Pulse Sensing (TRPS) analysis of EVs isolated from nMSCs and iMSCs, demonstrated two homogeneous populations with particle size mostly below 100 nm, with no significant differences between naïve EVs (nEVs) and induced EVs (iEVs) (**Figures 1A, B**). Transmission electron microscopy (TEM) identified EVs as a group of heterogeneous spheroids with the size ranging from 30 to 100 nm (**Figures 1D, E**). No apoptotic bodies were detected (28). Notably, EV particles/cell was increased by approximately three-fold in the medium of cytokine-conditioned MSCs (**Figure 1C**).

The immunomodulatory activity of EVs was evaluated *in vitro* by analyzing their dose-response effects on the production of the anti-inflammatory cytokine IL-10 by LPS-stimulated macrophages. Three different doses were tested (5,00E+7, 5,00E+8, 5,00E+9 nanoparticles respectively). Both nEVs and iEVs significantly increased IL-10 secretion after LPS treatment at the highest dose (**Figure 1G**). The effect of iEVs on angiogenesis *in vitro* was analyzed with a tube formation assay using the highest EV dose of the previous immunological test. The anti-angiogenic effect of iEVs is shown in **Figure 1H**: segment length and meshes area were drastically decreased in iEV treated cells. Representative pictures of the tube morphology derived from nEV and iEV treatment are shown in panel 1I.

EVs but Not MSCs Administration Ameliorates Clinicopathological Signs of Colitis

Treatment with DSS was administered in drinking water for 6 days. MSCs were injected at days 4 and 8 (**Figure 2A**) and EVs at days 4, 6 and 8 (**Figure 3A**). MSC dose was in the order of 10^6, similar to the one shown to be effective in previous publications on the treatment of DSS-induced colitis (29–31). We observed that the administration of both nMSCs and iMSCs worsened the typical signs of colitis evidenced by weight loss and colon length reduction with a trend to increased DAI (**Figures 2B–E**). Macroscopic examination of colons showed strong hyperemia and inflammation associated with the decrease in colon length (**Figures 2F, G**). Histological analysis of colon mucosa showed patchy areas of inflammatory infiltrate with tissue necrosis and ulcer formation. In line with these results, the severity of histological injury was increased in MSC-treated groups (**Figures 2H, I**).

With regards to the treatment with EVs, we set the doses in our previous work (32). We found that nEVs did not ameliorate body weight loss and colon length reduction, although it resulted in a modest but significant improvement in DAI (**Figures 3B–E**). Histomorphometric analysis did not show any appreciable effect of nEVs on colon mucosa (**Figure 3I**). However, treatment with iEVs was associated with normalization of body weight and with a significant improvement in DAI, although with a non-significant change in colon length (**Figures 3F, G**). Moreover,

TABLE 3 | Primer list.

Primers	Forward	Reverse	NM	Product length (bp)
NOS2	GCAGGTCTTTGACGCTCGGA	ATGGCCGACCTGATGTTGCC	NM_010927.3	105
Arg1	AGACCACAGTCTGGCAGTTGG	AGGTTGCCCATGCAGATTCCC	NM_007482.3	136
CD31	AGCCTCACCAAGAGAACGG	GTGGGGACAGGCTCATAAATAC	NM_001032378.2	150
Ang1	CAGTGGCTGCAAAAACTTG	AGACTGGTTCCTATCTCAAGC	NM_001286062.1	119
Vegf	CTCCACCATGCCAAGTGGTC	GTCCACCAGGGTCTCAATCG	NM_001025250.3	126
B2micro	GCTTCAGTCGTCAGCATGG	CAGTTCAGTATGTTCGGCTTCC	NM_009735.3	149

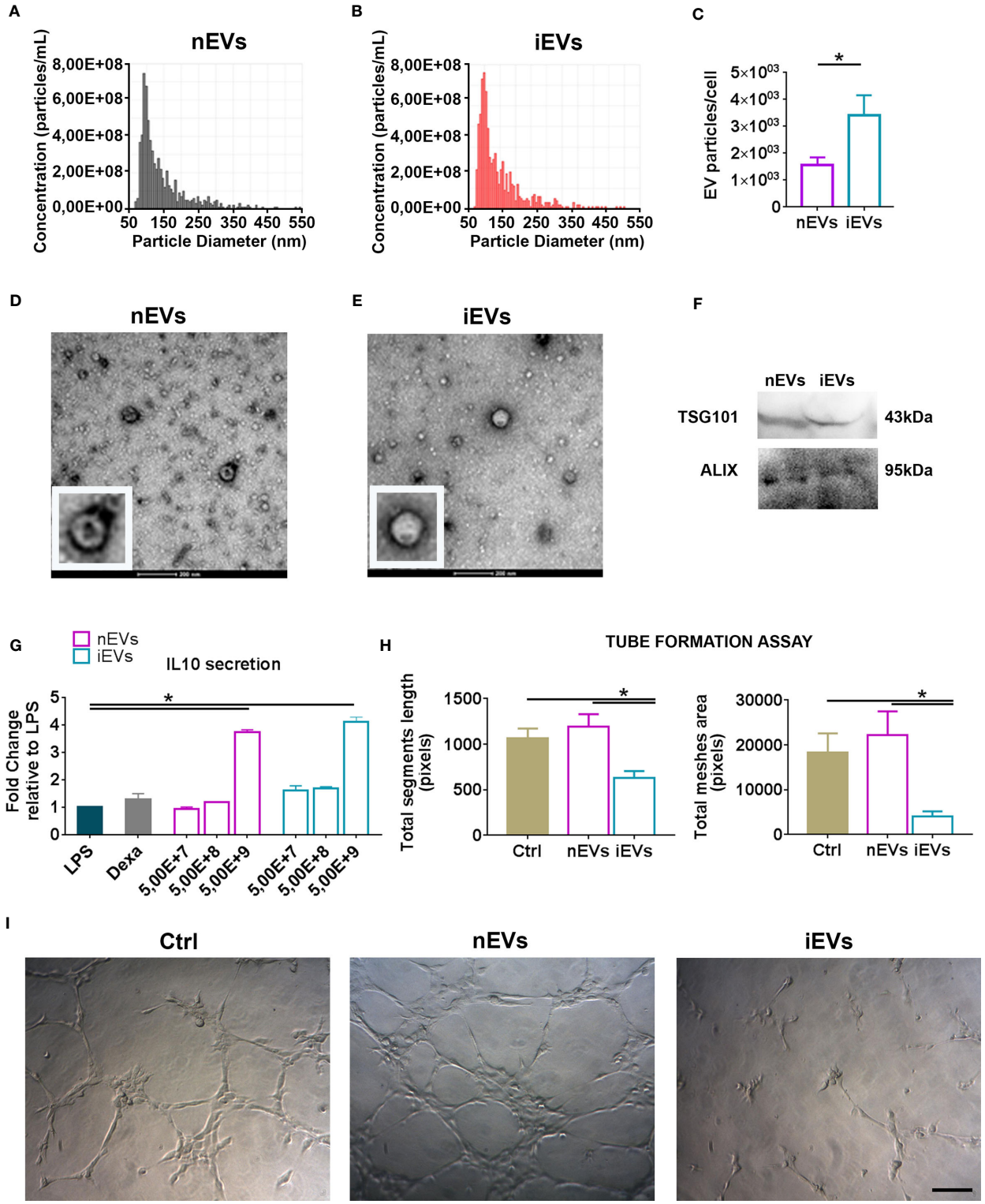

FIGURE 1 | Characterization of extracellular vesicles derived from mesenchymal stromal cells. **(A)** Representative size distribution of nEV and of **(B)** iEV analyzed by Resistive Pulse Sensing. **(C)** Number of nEVs and of iEVs secreted per cell. Result are mean ± standard error (n = 5 independent experiments, *P < 0.05). **(D)** Transmission electron microscopy analysis of freshly nEVs **(E)** and of freshly iEVs; scale bar refers to 200 nm. **(F)** Western Blot of nEVs and iEVs for TSG101 and Alix. **(G)** Macrophage functional assay. IL-10 quantification after LPS cell treatment. (n = 3 independent experiments, *P < 0.05). **(H, I)** Endothelial functional assay. Segment length and meshes area were measured after nEVs and iEVs treatment. **(I)** Representative phase contrast images. Scale bar: 100μm.

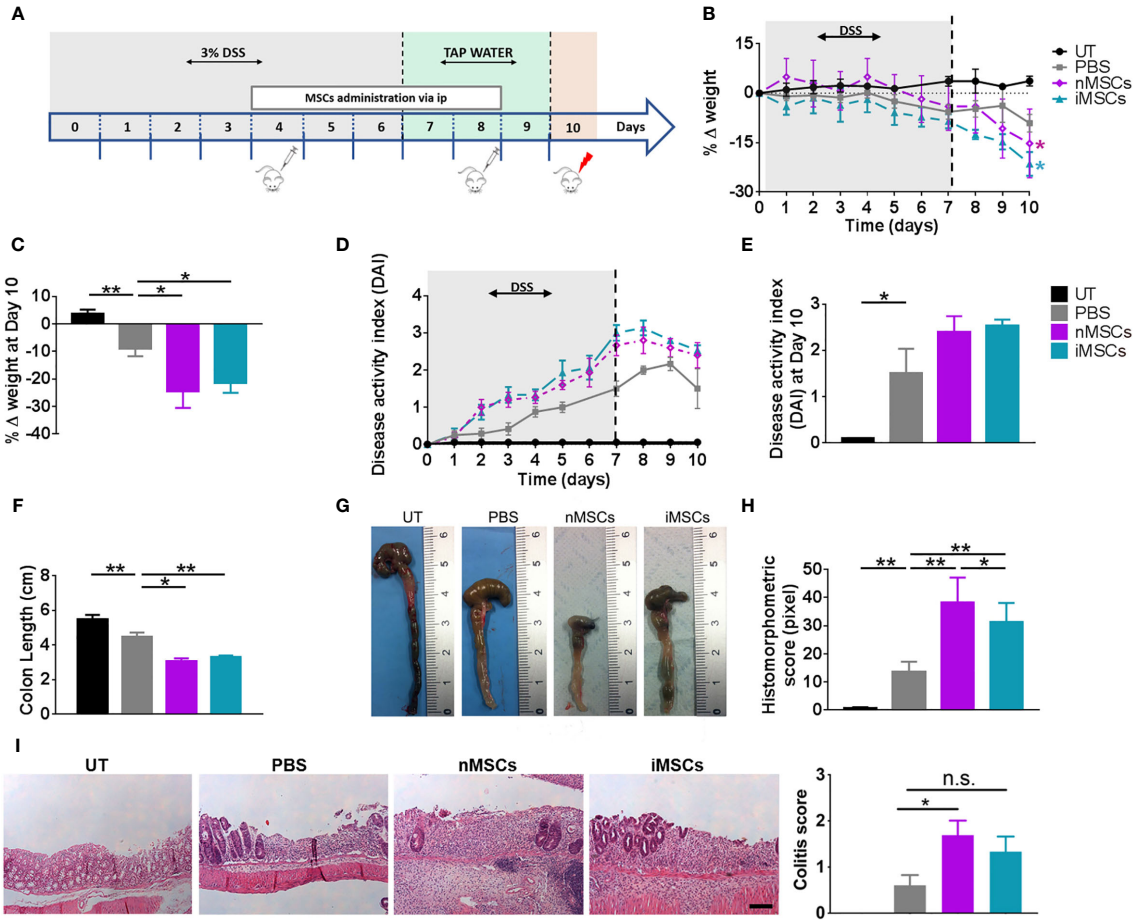

FIGURE 2 | MSC administration worsens colitis in mice. **(A)** Experimental design. DSS was given in drinking water for 6 days followed by three days on plain water. MSCs were injected intraperitoneally on days 4 and 8. Weight loss **(B)**, Percent weight loss **(C)** and disease activity scores **(D)** were determined daily. **(E)** Disease activity index at 10 days. Colon length **(F, G)**, histomorphometric score **(H)** and colitis score **(I)** were determined on day 10. Scale bars, 50 μm. Results are mean ± standard error (n = 5). *P < 0.05, **P < 0.01, n.s. = not significant.

the histomorphometric and colitis score analysis confirmed the beneficial effect of iEVs evidenced by a strong reduction of necrotic mucosal surface (**Figures 3H, I**).

Different Effects of Unprimed or Cytokine-Primed MSCs and of Their Secreted EVs on Intestinal Macrophage Polarization

Induction of colitis was associated with an increased infiltration of $CD45^+$ cells in colon tissue. This parameter did not show significant differences between the different treatments (nMSC vs iMSC and nEVs vs iEVs) (**Figures 4A** and **5A**). Specifically, nMSCs administration tended to induce an anti-inflammatory polarization in macrophages. This effect was statistically significant when analyzing the Nos2/Arg1 ratio by qRT-PCR (**Figure 4D**) and there was a trend when analyzing $iNOS^+$/$CD163^+$ cell ratio by immunofluorescence (**Figure 4E**). Conversely, the administration of iMSCs polarized macrophages to a pro-inflammatory phenotype. Indeed, both the ratio of the genes *Nos2/Arg1* and of the proteins $iNOS^+$/$CD163^+$ cell ratio was significantly higher (**Figures 4B–E**). No effect of MSC administration (either naïve or induced) was observed on the Treg/Teff ratio at the level of the intestinal lymph node as the ratio were similar to that of the PBS group (**Figure 4F**). With regards to the administration of nEVs, they did not show a clear effect on macrophage polarization nor on Treg/Teff ratio, although a reduction of iNOS expression (M1 marker) was observed by immunofluorescence of colon tissue (**Figures 5B–F**). However, administration of iEVs promoted macrophage polarization to an anti-inflammatory phenotype, as shown by a decrease in both the *Nos2/Arg1* ratio and $iNOS^+$/$CD163^+$ cell ratio. In particular, immunofluorescence analysis showed a significant reduction of the M1 marker iNOS and a significant increase of the M2 marker CD163 in colon tissue of iEV-treated animals (**Figures 5B–E**). This finding was associated with a significant increase in the Treg/Teff ratio at the level of the intestinal lymph node (**Figure 5F**).

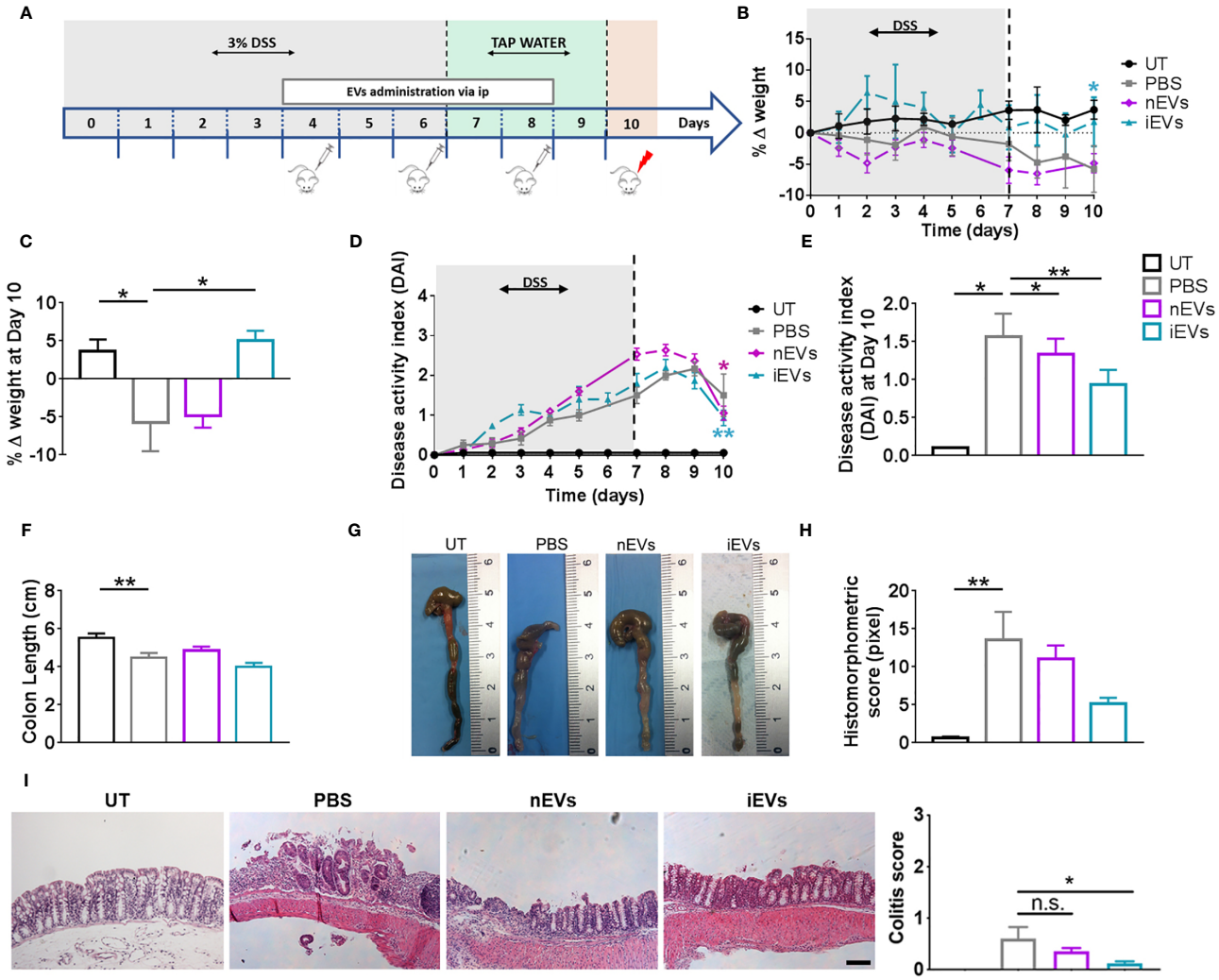

FIGURE 3 | EVs improve the clinicopathological signs of colitis. **(A)** Experimental design. DSS was given in drinking water for 6 days followed by three days on plain water. EVs were injected intraperitoneally on days 4, 6 and 8. Weight loss **(B)**, Percent weight loss **(C)** and disease activity scores **(D)** were determined daily. **(E)** Disease activity index at 10 days. Colon length **(F, G)**, histomorphometric score **(H)** and colitis score **(I)** were determined on day 10. Scale bars, 50 μm. Results are mean ± standard error (n = 5). *P < 0.05, **P < 0.01, n.s. = not significant.

Different Effects of MSCs and of Their Secreted EVs on Collagen Deposition and Angiogenesis

Both nMSCs and iMSCs increased collagen deposition in colon mucosa, however statistical significance was achieved only with the administration of the latter (**Figure 6A**). The presence of the endothelial marker CD31 was used to assess angiogenesis by qRT-PCR and immunocytochemistry. CD31 expression was increased at protein level, although to a significant extent only in the group treated with unprimed MSCs (**Figure 6B**). Gene expression of CD31, *Ang1* and *Vegf* was down regulated in both naïve and primed MSCs groups (**Figure 6C**). Conversely, both collagen deposition and angiogenesis were significantly reduced in the iEV-treated group. In the same group of mice, CD31, *Ang1* and *Vegf* gene expression decreased as well as CD31 detected as protein in the colon tissue. nEVs administration had no effect on both parameters (**Figures 6D–F**). This finding suggests that iEVs are best suited as an anti-angiogenic agent.

Effects of MSCs and of Their Secreted EVs on Mucin 5ac in Colon Mucosa

In order to investigate the intestinal mucosa at functional level, the expression of mucin 5ac was analyzed. The treatment with DSS significantly reduced mucin 5ac expression, suggesting loss of functionality. However, this protein increased in colon mucosa of mice receiving unprimed MSCs in respect to PBS treated animals (**Figure 7A**), while iMSCs had no effect (**Figure 7A**). Of note, a strong 8-fold induction of MUC5ac was observed in mice receiving iEVs, suggesting a regenerative action of iEVs, yet the administration of nEVs had no significant effect (**Figure 7B**).

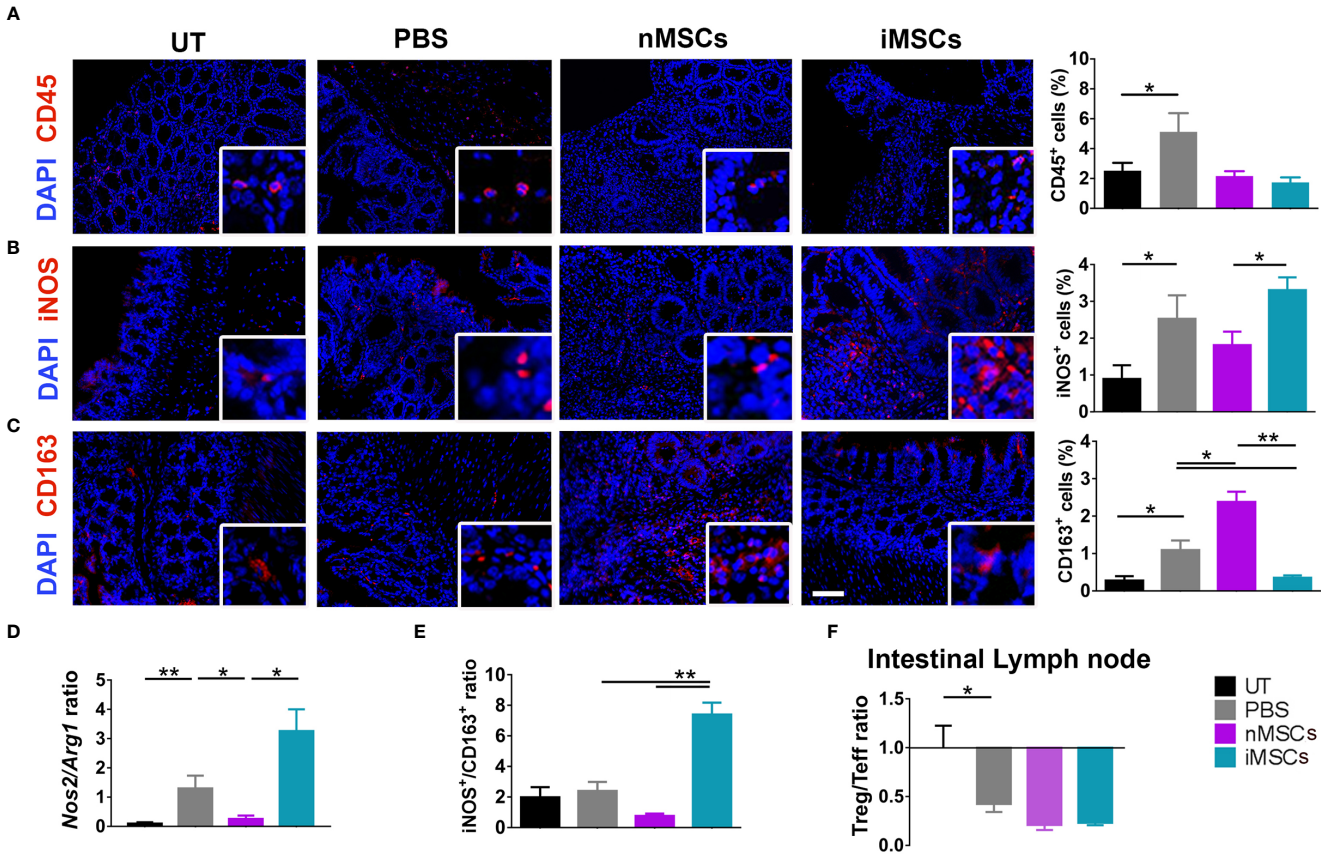

FIGURE 4 | Administration of cytokine-conditioned MSCs is associated with pro-inflammatory polarization of intestinal macrophages. **(A)** CD45 pan lymphocyte IF. CD45 positive cells are equally present in nMSCs and iMSCs treated mice. **(B, C)** Immunofluorescence labeling of macrophage polarization markers in colon mucosa: IF (left), quantification (right). iMSCs induce iNOS protein, M1 marker. **(D)** qRT-PCR M1/M2 ratio: iEVs administration results in a significant presence of anti-inflammatory *Arg1* gene. **(E)** protein M1/M2 ratio. **(F)** Treg/Teff ratio in the intestinal lymph node. Scale bar: 50μm. Results are mean ± standard error (n = 5). *P < 0.05, **P < 0.01.

DISCUSSION

Chronic intestinal inflammation results from the interaction of genetic, immunological and environmental factors. Recently, Mesenchymal Stromal Cells (MSCs) have been approved by the European Medicines Agency for the treatment for Crohn's fistulas resistant to conventional therapy and biologics. The effectiveness of MSC therapy probably relies on its complex immune regulatory rather than suppressive properties, being able of redirecting both the innate and the adaptive and innate immune system towards an anti-inflammatory, pro-regenerative response. However, cell dosages and timing are still based on insufficient experience and may be limited by high production costs (33). Even more critical, administered MSCs may behave differently in different patients. MSCs secrete soluble factors such as indoleamine 2,3-dioxygenase (IDO), involved in the catabolism of the essential amino acid tryptophan (34). This results in the degradation of tryptophan and accumulation of toxic kynurenines with inhibition of T cell proliferation. Upregulation of stress response pathways such as inducible nitric-oxide synthetase (iNOS) variably contributes to MSCs-induced immune suppression, with notable species differences (5). During MSCs-mediated immunomodulation, proinflammatory cytokines have been shown to play a key role, provoking MSCs to express iNOS (in rodents) or IDO (in humans) associated with T cell suppression. Waterman et al. (7) described a different immunomodulatory mechanism of MSCs that can polarize to a proinflammatory or to an immunosuppressive phenotype depending respectively on Toll-like receptor (TLR) 4 or TLR3 priming, thus again depending on the characteristics of the inflammatory environment. These findings suggest that it is important to be aware of the potential differential effects of cytokines or drugs on the expression and activity of IDO when applying MSCs in the treatment of disease, as they are a critical switch that determine the immunomodulatory fate of MSCs.

Several reports confirmed the immunomodulatory and pro-regenerative effects of MSCs and their secreted EVs. However, we obtained strikingly different results in our well-established murine model of DSS-induced colitis. In this experimental setup, administration of MSCs (both unprimed and cytokine-conditioned) worsened the clinical signs of colitis, as shown by a decreased body wt and by a trend to an increased DAI.

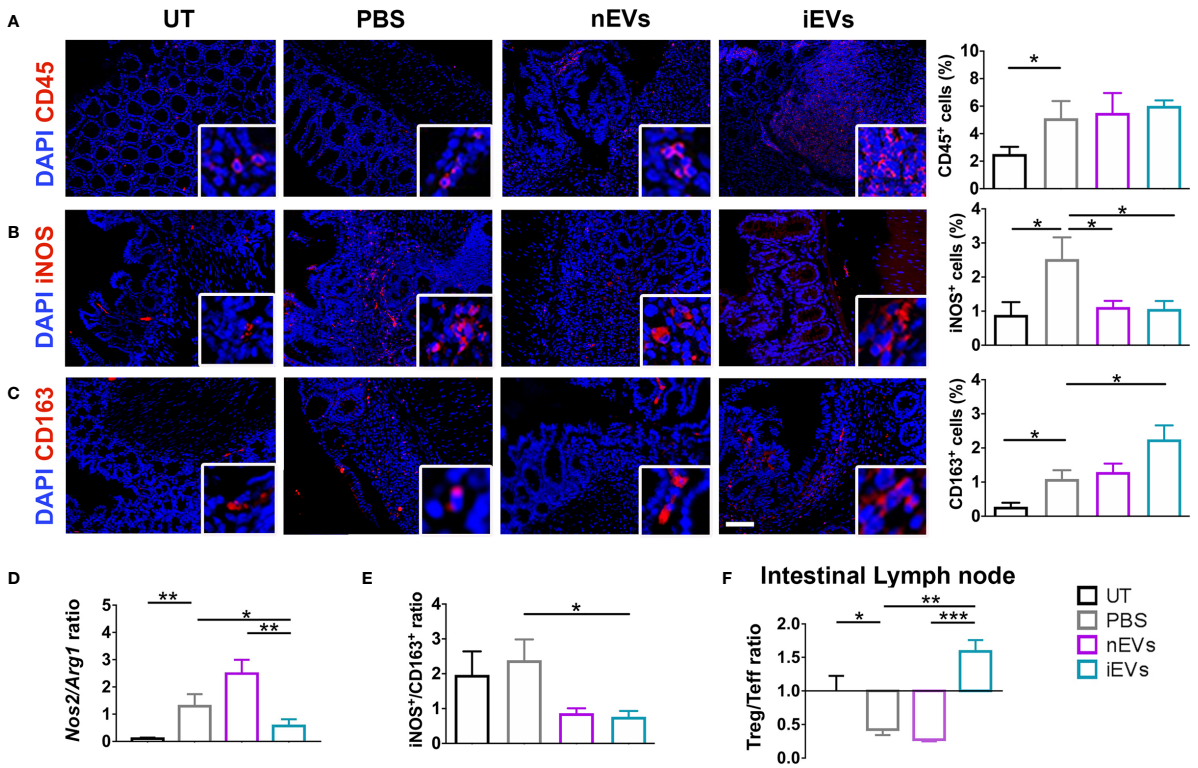

FIGURE 5 | EV administration reverts macrophage polarization to an anti-inflammatory phenotype and improves M1/M2 and Treg/Teff ratio. **(A)** CD45 pan lymphocyte IF. CD45 positive cells are equally present in PBS, nEVs and iEVs treated mice. **(B, C)** Immunofluorescence labeling of macrophage polarization markers in colon mucosa: IF (left), quantification (right). iEVs induce CD163 protein, M2 marker. **(D)** qRT-PCR M1/M2 ratio: iEV administration results in a significant presence of anti-inflammatory *Arg1* gene. **(E)** protein M1/M2 ratio. **(F)** Cytofluorimetric analyses of freshly isolated cells form intestinal lymph node: Treg/Teff ratio. Scale bar: 50μm. Results are mean ± standard error (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001.

Moreover, the histomorphometric scores of colitis were significantly worsened in both MSC-treated groups. It was reported that the therapeutic efficacy of MSCs in experimental colitis could be improved by priming with pro-inflammatory cytokines (35, 36). We have thus treated a group of mice with MSCs conditioned with a cytokine cocktail (iMSCs) known to enhance the cell immunomodulatory and anti-angiogenic activity (20, 21). However, iMSC administration also worsened colitis compared with the PBS-treated (control) group. Even if several reports describe the therapeutic efficacy of MSCs in DSS-induced colitis (29–31, 37–39) a worsening effect of these cells in the present model was reported by other investigators (40). It was also reported that MSCs can revert to a pro-inflammatory phenotype in collagen-induced arthritis (41, 42). It should be noticed that we used murine (syngeneic) MSCs in order to avoid possible bias due to species differences, as in the work of Nam et al. (40). However, improvement of DSS-induced colitis was described following administration of syngeneic (31, 43), allogeneic (37, 38) and xenogeneic (human) MSCs (29). The opposite effects observed by us and by Nam et al. are probably resulting from the poor reproducibility of the experimental model that can be influenced by strain differences and by environmental factors, the latter possibly affecting the composition of intestinal microbiota and thus the local immune response (44, 45). However, our results confirm that the effect of exogenous MSCs can be unpredictable, likely depending on poorly understood host environmental factors.

Administration of EVs secreted by unprimed nMSCs was not effective in ameliorating clinical and histomorphometric indexes of colitis, although a trend to improvement was present both in the DAI and in the histomorphometric score. However, administration of iEVs, obtained from cytokine-conditioned MSCs, was associated with a significant improvement in all clinicopathological parameters of disease.

The effects of cytokine priming on MSCs and the composition of iEVs vs. nEVs were characterized previously (23, 46). MSC stimulation with the cytokine cocktail described here resulted in the enhanced secretion of several specific proteins involved in inflammation and angiogenesis (46), including matrix metalloproteases (MMPs), which are considered both effectors and regulators of several biological processes since they can activate, inactivate or antagonize growth factors, cytokines and chemokines by proteolytic processing. In a more recent study, it was demonstrated that cytokine-stimulated MSCs produce EVs that are enriched in the anti-angiogenic metalloprotease TIMP-1 (23). Moreover, these iEVs carry CD39 and are particularly enriched in CD73 (23). Since the combination of CD39 and CD73 degrade ATP, ADP, and AMP to adenosine, these iEVs

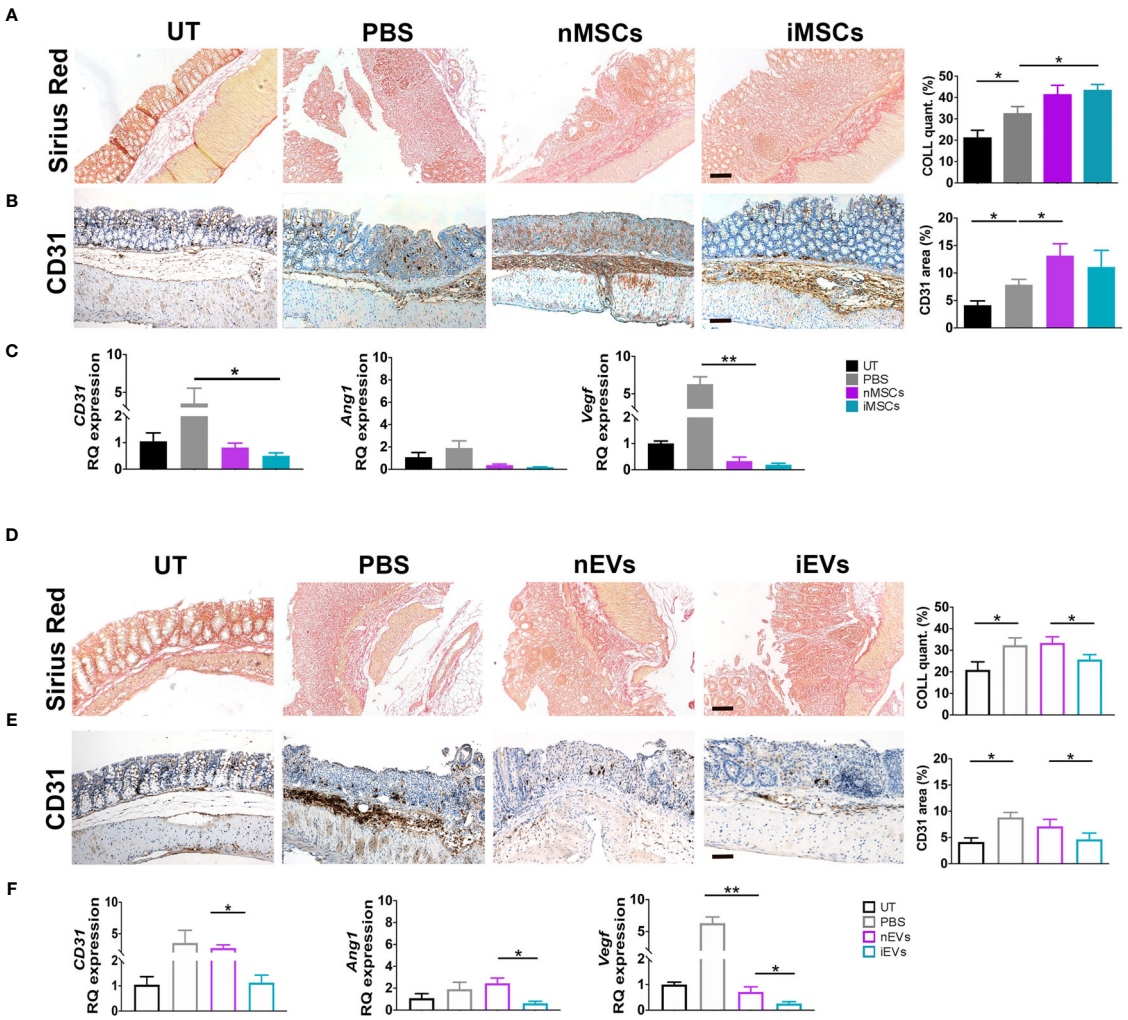

FIGURE 6 | MSCs and iEVs exert opposite effect on fibrogenesis and angiogenesis in mice with colitis. **(A)** Collagen deposition in colon mucosa: Sirius red (left), quantification (right). **(B)** Quantitative CD31 staining as a measure of angiogenesis in colon mucosa: immunocytochemistry (left), quantification (right). **(C)** qRT-PCR of *CD31, Angiopoietin, Vegf* genes. All the genes were down regulated in nMSCs and iMSCs injected mice. **(D)** Collagen deposition in colon mucosa: Sirius red (left), quantification (right). **(E)** Quantitative CD31 staining as a measure of angiogenesis in colon mucosa: immunocytochemistry (left), quantification (right). **(F)** qRT-PCR of *CD31, Angiopoietin, Vegf* genes. With the exception of *CD31* in nEVs, *Angiopoietin* and *Vegf* were down regulated in nEVs and iEVs injected mice. Scale bar: 50μm. Results are mean ± standard error (n = 5). *P < 0.05, **P < 0.01.

might be viewed as "immunological switches" that shift ATP-driven pro-inflammatory immune cell activity toward an anti-inflammatory state mediated by adenosine (47). Extracellular adenosine reduces the expression of adhesion molecules in endothelial cell, thus preventing the recruitment of leukocytes into the injured tissue (48, 49). Angioni et al. (23) also identified a novel anti-angiogenic mechanism of iEVs based on adenosine production, triggering of A2B adenosine receptors, and induction of NOX2-dependent oxidative stress (leading to apoptosis) within endothelial cells. In the present work we confirmed the anti-angiogenic effect of iEVs in an *in vitro* tube formation assay and demonstrated that these nanoparticles can also polarize macrophages to an anti-inflammatory phenotype *in vitro* (**Figure 1**). Thus, MSC stimulation with the present cytokine cocktail results in the production of EVs targeting both inflammation and angiogenesis at several levels.

Previous reports in animal models of experimental colitis (50, 51) described beneficial effects of allogeneic (rat) or xenogeneic (human to mice) bone marrow-derived MSC-EVs. We cannot exclude that the trend to improvement observed in our nEV treated group could become significant with higher EV dosages. MSC dose in mice was in the order of 10^6, similar to the one shown to be effective in previous publications on the treatment of DSS-induced colitis (29–31). As described in the manuscript, in choosing the EV dose we empirically applied the cell/EV ratio of 1:1000. This EV dose per Kg body wt was shown to be effective in animal models of lung injury in previous publications by others and by us (32, 52, 53) and in a patient with GVHD (54).

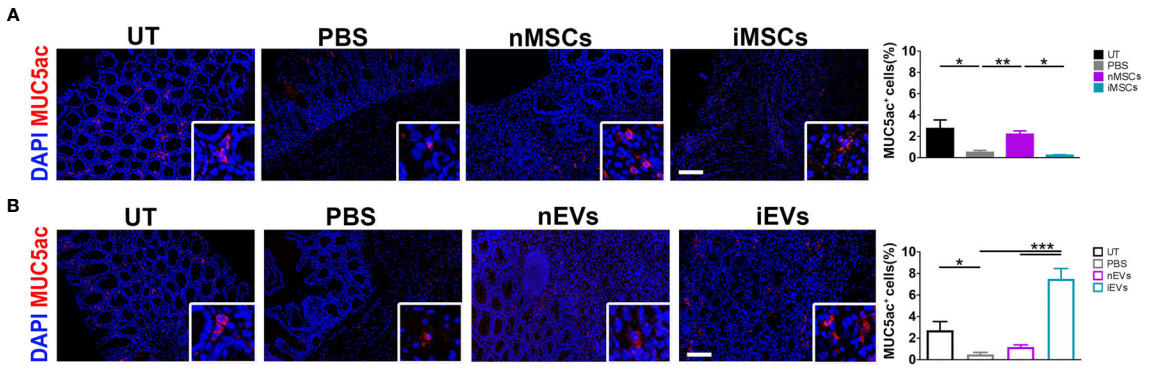

FIGURE 7 | iEVs strongly enhance Mucin 5ac in mice with colitis. **(A)** Quantification of MUC5ac in colon mucosa in nMSCs and iMSCs mice: IF (left), quantification (right). **(B)** Quantification of mucin 5ac in colon mucosa in nEVs and iEVs mice: IF (left), quantification (right). Results are mean ± standard error (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001.

However, we clearly demonstrated that, at similar dosages, the therapeutic efficacy of EVs is greatly enhanced by cytokine preconditioning of the parent cells. More importantly, we showed that MSC-EVs exert a beneficial effect in the same pathologic environment that is on the contrary adversely affected by administration of their parent cells. To our knowledge, this is the first *in vivo* demonstration that EVs can produce opposite effects with respect to their cells of origin. Moreover, these findings suggest that the effects of EVs can be more reproducible than their parent cells. Indeed, unlike cells, EVs carry a definite set of signals, which should not be modified by exposure to host environmental factors.

Colitis induced by short-term DSS administration is an acute model of mucosal inflammation involving innate immune mechanisms, resulting in macrophage and neutrophil infiltration (45). Therefore, this model does not mirror the complexity of chronic IBD. However, the central role of macrophages in the pathogenesis of IBD is well recognized (39, 55). The expression of iNOS2, a molecular marker of M1 activation, was increased following induction of colitis and remained high in the two MSC- treated groups, consistently with the clinicopathological data. The expression of CD 163 as a marker of M2 activation was more variable, showing an increase in the nMSC-treated group and a decrease in the iMSC-treated mice. An opposite behavior was again observed in the EV-treated groups: the M1 activation marker was significantly decreased in both nEV-treated and iEV-treated mice, and the M2 activation marker was significantly increased in the iEV-treated mice. Clearly, the complexity of the macrophage activation states cannot be fully described by changes in the above molecular markers. However, such changes are consistent with the clinicopathological improvements observed in EV-treated mice and with lymphocyte profile in the intestinal lymph node. As discussed above, nMSCs tended to polarize the macrophages to an anti-inflammatory phenotype. However, only EVs suppressed the expression of iNOS, a major effector of inflammation. This observation might help explaining the differential effects of MSCs and of their secreted EVs on the Treg/Teff ratio and on clinical and histological findings. Clearly, the mechanisms underlying the effect of EVs on iNOS are currently unknown and require further investigation. The development of colitis was associated with a reduced Treg/Teff ratio, a finding that was not modified in the groups treated with MSCs. The reduction of the Treg/Teff ratio was also unaffected by nEV administration, but was reversed in iEV-treated mice, consistently with the increased M2 macrophage polarization observed in this group.

Inflammation, angiogenesis and collagen deposition that, with time, strengthen fibrogenesis, are intimately intertwined processes. Dysregulated angiogenesis is a hallmark of pathological processes such as chronic inflammation and cancer (56, 57). Our results with high expression of CD31 in nEVs treated mice and low expression of the angiogenic protein in iEVs animals, suggest that the increased therapeutic efficacy of iEVs compared to nEVs in the present experimental model is probably multifactorial. The apparent inconsistency between CD31% area and RQ expression (**Figure 6**) could be explained with a diminished gene expression in this time window, i.e. 3 days after interrupting DSS administration, while the translated protein is still present in the tissue, We have recently observed that EVs secreted by MSCs primed with IL1β, IL6 and TNFα exhibit increased expression of CD73 (23). CD73 acts in concert with CD39 to degrade ATP, ADP, and AMP to adenosine, shifting the ATP-driven pro-inflammatory immune response toward an anti-inflammatory condition mediated by adenosine (47). Therefore, amelioration of colitis could be at least partially mediated by such enhanced anti-inflammatory activity of iEVs. However, we also observed that iEVs exert a strong anti-angiogenic activity mediated by the expression of TIMP-1 peptide (23), consistently with the concept that inflammation and angiogenesis are intimately interlinked (57, 58). In particular, angiogenesis is an important component in the pathogenesis of IBD (59) and anti-angiogenic therapies have been used in this disorder (60). Moreover, it was shown that treatment with anti-angiogenic peptides ameliorates DSS-induced colitis (61, 62). The anti-angiogenic activity of iEVs could thus represent an additional mechanism responsible for the therapeutic effects observed in the present work.

Tissue fibrosis, with connective tissue replacing normal parenchymal tissue, can result from excessive or dysregulated inflammation (63). Scar formation resulting in intestinal obstruction is a major complication In Crohn's disease (64). Collagen deposition was increased following induction of colitis and was further increased in MSC-treated mice. On the contrary, iEV-treated animals showed diminished collagen, likely resulting from the reduced inflammatory injury observed in this group.

The mucus layer lining the epithelial surface of the intestine has several functions, including the control of the passage of nutrients and the regulation of host interactions with the intestinal microbiota (65, 66). Secreted mucins are the major proteins found in the mucus layer, with MUC2 being largely predominant in the healthy intestinal mucosa, while MUC5AC (human)/Muc5ac (murine) is alternatively secreted in IBD and in experimental colitis (67). Previous studies both in mice and in patients with IBD suggest that Muc5ac/MUC5AC expression in the gastrointestinal tract is a tissue-protective response during active inflammation (67, 68). Here, we show that MUC5ac was reduced following the induction of chemical colitis, probably because of mucosal cell destruction and of the detergent effect of DSS on the mucus layer. Interestingly, administration of iEVs resulted in a striking 8-fold increase of Muc5ac, likely because of reduced epithelial injury and increased mucus secretion. Increased mucus secretion could help restoring the intestinal barrier, thus contributing to the observed reduction in mucosal inflammation. Regulation of MUC5ac secretion is poorly understood (67). Therefore, the mechanisms underlying these novel findings are presently unknown and require further investigation. Of note, nMSC administration was also associated with a much lower but significant increase in MUC5ac production. It thus seems that nMSCs do exert some anti-inflammatory and pro-regenerative effects, but such effects seem insufficient to overcome the strong inflammatory condition associated with this animal model, as reported by others (40).

We should however emphasize that the present work compares very different products. Since the composition of secreted EVs is dependent on the response of the cells to the environment (69), the EVs produced by MSCs *in vitro* are likely quite different from those produced by the same cells *in vivo*. Moreover, it is known that most exogenous cells will die within hours from transplantation (70–72), further affecting both the quality and the quantity of secreted EVs.

A major limitation of this study is the lack of a dose-response assessment. In particular, administration of higher EV doses is required to define the therapeutic range and to explore possible side effects.

In summary, we show for the first time that EVs derived from cytokine-primed MSCs can exert beneficial effects *in vivo* in the same inflammatory condition that is on the contrary adversely affected by their cells of origin. Our findings also suggest that the effects of nEVs could be more reproducible with respect to their cells of origin, supporting the concept of a better safety profile in favor of these nanoparticles (73). In the present model, EVs confirmed their pleiotropic effects, affecting several different immunological pathways. However, additional mechanistic studies are needed to dissect the complex mode of action of these nanoparticles in inflammatory conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Italian Ministry of Health, Division of Veterinary Medicine (protocol n°861/2016-pr, risp. a prot c35de.2 #195042387#).

AUTHOR CONTRIBUTIONS

AMT, MPo, MM: conception and design of the experiments, collection and assembling of the data, data analysis and interpretation, manuscript writing. IC, MPi, MG, CF. conception and design of the experiments, collection and assembling of the data. FM, GDL, RM, FC, MS: collection and assembling of the data. VM, RDC, IA, AV, AP: analysis and interpretation of the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.627605/full#supplementary-material

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Exosome-Contained APOH Associated With Antiphospholipid Syndrome

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Background: Antiphospholipid syndrome (APS) is a systemic autoimmune disease that can lead to thrombosis and/or pregnancy complications. Exosomes, membrane-encapsulated vesicles that are released into the extracellular environment by many types of cells, can carry signals to recipient cells to affect angiogenesis, apoptosis, and inflammation. There is increasing evidence suggesting that exosomes play critical roles in pregnancy. However, the contribution of exosomes to APS is still unknown.

Methods: Peripheral plasma was collected from healthy early pregnancy patients (NC-exos) and early pregnancy patients with APS (APS-exos) for exosome extraction and characterization. The effect of exosomes from different sources on pregnancy outcomes was determined by establishing a mouse pregnancy model. Following the coincubation of exosomes and human umbilical vein endothelial cells (HUVECs), functional tests examined the features of APS-exos. The APS-exos and NC-exos were analyzed by quantitative proteomics of whole protein tandem mass tag (TMT) markers to explore the different compositions and identify key proteins. After incubation with HUVECs, functional tests investigated the characteristics of key exosomal proteins. Western blot analysis was used to identify the key pathways.

Results: In the mouse model, APS-exos caused an APS-like birth outcome. In vitro experiments showed that APS-exos inhibited the migration and tube formation of HUVECs. Quantitative proteomics analysis identified 27 upregulated proteins and 9 downregulated proteins in APS-exos versus NC-exos. We hypothesized that apolipoprotein H (APOH) may be a core protein, and the analysis of clinical samples was consistent with finding from the proteomic TMT analysis. APOH-exos led to APS-like birth outcomes. APOH-exos directly enter HUVECs and may play a role through the phospho-extracellular signal-regulated kinase pathway.

Conclusions: Our study suggests that both APS-exos and APOH-exos impair vascular development and lead to pregnancy complications. APOH-exos may be key actors in the pathogenesis of APS. This study provides new insights into the pathogenesis of APS and potential new targets for therapeutic intervention.

Keywords: antiphospholipid syndrome, exosomes, proteomics analysis, mice model, MAPK pathway

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INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by thrombosis and/or pregnancy complications due to persistent (≥12 weeks) medium/high titers of antiphospholipid antibodies (aPLs) (1, 2). Catastrophic APS may lead to widespread small-vessel thrombosis and multiorgan failure, with a mortality rate of more than 50% (3). APLs, including IgG and IgM of anti-b2-glycoprotein I antibodies (also known as anti-b2-GPI; anti-APOH) and/or cardiolipin (aCL), and /or lupus anticoagulant, which have a clear correlation with the clinical manifestations of APS, but the pathophysiology between them is not clear (1, 4). The major target of aPLs is apolipoprotein H (APOH, also known as β2-glycoprotein I, β2GPI), a plasma protein that binds avidly to phospholipid surfaces, especially when dimerized by binding to aPLs (5–9). Endothelial dysfunction is a key pathological component of APS (10). The pathological manifestations of APS are vascular remodeling, angiogenesis injury and endothelial cell injury. Long-term anticoagulant therapy is the only potent treatment for APS. However, it is not effective in all patients and increases the risk of bleeding (5). There is an urgent need to explore the etiology of APS.

Exosomes are saucer-like vesicles with diameters of approximately 30–150 nm. They are released from various cell types, such as fetal cells, immunocytes and tumor cells (10–12). Studies have suggested that exosomes and their contents (i.e., proteins, mRNAs, and microRNAs) contribute to the regulation of multiple biological processes during pregnancy (12, 13). Accumulating evidence supports the hypothesis that exosomes play significant roles in the pathophysiology of inflammation, thrombosis and autoimmune diseases (14, 15). In the pathology of APS, exosomes may injure the vascular endothelium function as an independent mechanism. (16). To date, little is known about the role exosomes play in APS during pregnancy.

In this study, we provide novel evidence that APS-exosomes (exos) and APOH-exos are associated with adverse reproductive events in a mouse model, likely facilitated by impaired endothelial cell functions.

MATERIALS AND METHODS

Ethics Statement

From July 2018 to December 2018, plasma samples from healthy pregnant women and pregnant women with APS were collected at the First Maternal and Infant Health Hospital Affiliated with Tongji University (n = 6 per group, 12 in total). Three umbilical cords were obtained from healthy newborns at the same hospital. Approval was obtained from the Institutional Ethics Committee of the Shanghai First Maternal and Infant Hospital affiliated with Tongji University (KS18128). All mothers provided written informed consent and were of the same age and gestational age ranges.

The current study used plasma (time of sampling was between 6–8 weeks of gestation). Blood was drawn into vacutainer tubes containing sodium citrate (0.129 M). Plasma was obtained by centrifugation of the blood at 3000 × g for 5 min at 4°C and frozen at −80°C (Thermo Scientific, USA) until analysis.

Extraction of Exosomes by Ultracentrifugation

Exosomes were initially extracted from the plasma samples of healthy pregnant women (NC-exos), pregnant women with APS (APS-exos) and cell supernatant by 3000 × g centrifugation at 4°C for 5 min to remove cells and cellular debris, followed by 10000 × g centrifugation at 4°C for 60 min (Avanti J-26S XP). The supernatant was combined with phosphate buffered saline (PBS, Biological Industries, Israel) and then centrifuged at 100000 × g at 4°C for 120 min (OptimaXE-90 Ultracentrifuge). The exosome pellets were resuspended in PBS and stored at −80°C.

Transmission Electron Microscopy (TEM)

To ascertain the purity of isolated exosomes, the samples (approximately 10 μg/ml) were fixed in 1% (final concentration) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After attachment to TEM grids, the exosomes were negatively stained with 1% uranyl acetate. Images were taken from different regions of the grid.

Nanoparticle Tracking Analyzer (NTA)

The sample cells were rinsed with deionized water, and the analyzer was calibrated with polystyrene microspheres (110 nm). After the sample cells were washed with PBS, samples were diluted in PBS and analyzed in triplicate.

Lentiviral Transduction

Lentivirus was provided by the Hanyin Biotechnology Limited Company (Shanghai). The carrier component was CMV-MCS-EF1α-ZsGreen1-PGK-puro. To demonstrate the effects of APOH protein, we transduced human embryonic kidney cells (293T) cells with lentivirus overexpressing APOH (APOH-293T) or empty lentivirus (N-293T) as a control. After transduction, the APOH-exos and control (N)-exos were isolated from cell culture medium.

Immunofluorescence Microscopy

Human umbilical vein endothelial cells (HUVECs) were inoculated on glass-bottom dishes and cultured to 50% confluence. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil, Thermo Fisher) was added to the dishes to stain exosomes overnight. The cells were then fixed in 4% paraformaldehyde for 10 min and permeabilized in 0.1% Triton X-100 in PBS for 10 min at room temperature. After being washed and resuspended in PBS, the cells were incubated with phalloidin-FITC (Sigma, Darmstadt, Germany) for 30 min, washed in PBS, stained with 4′,6-diamidine-2-phenylindole (DAPI, Sigma) for 10 min, and washed again in PBS. Finally, the cells were placed on slides and covered with Fluoromount-GTM water-soluble compound. The slides were observed by confocal microscopy on an inverted microscope (TCS SP8; Leica, Weizlar), and fluorescence images were captured.

Isolating, Grouping and Processing of HUVECs

Residual blood was washed out with PBS, and the umbilical vein cavity was digested in type I collagenase (Sangon Biotech) diluted to 0.8% in Hanks balanced saline solution at 37°C for 15–20 min. Subsequently, the contents were quickly rinsed in a 50 ml centrifuge tube, centrifuged at 1500 × g for 3 min, and transferred to a 3 cm culture dish. After 24 h of cultivation, the medium was replaced. The third to sixth generations of HUVECs were used for all experiments.

Cell Culture

Using the conventional culture method (i.e., 37°C, 5% CO_2 and 100% relative humidity in an incubator), the HUVECs were incubated in endothelial culture medium containing 5% fetal bovine serum and 1% endothelial cell growth factor (ScienCell), and the 293Ts were incubated in high-glucose Dulbecco's modified eagle's medium (DMEM, HyClone) and placed in saturated humidity culture conditions. The 293Ts were incubated in high-glucose DMEM containing 5% fetal bovine serum (HyClone) until they reached 80%–90% confluence. Then, the 293Ts were cultured for 48 h in high-glucose DMEM containing 2% BSA, and the supernatant was collected.

Cell Proliferation, Migration and Tube Formation Assays

Proliferation, migration, and tube formation abilities of HUVECs were routinely evaluated. In brief, 3×10^3 cells per well were plated in 96-well plates, then different doses of exosomes were added. Cell numbers were assessed using cell counting kit-8 at 450 nm (Thermo Fisher Scientific). For migration, 8×10^4 cells were seeded in the upper chamber of a transwell chamber, and exosomes were added to the lower chamber. Following a 16 h incubation, hematoxylin staining was performed for each chamber. Migrated cells were counted in a transwell chamber (Corning BioCoat, USA). To quantitate *in vitro* tube formation, plates were coated with Matrigel (Corning BioCoat, 35420) substrate. HUVECs in serum-free endothelial cell medium were added with different doses of exosomes and photographed using an inverted microscope within 6 h. Images were taken at 40/100× magnification by optical microscopy. The above process was repeated three times, and the results were counted with ImageJ software.

Protein Extraction and Quantification

Exosomes collected form each group were added to a 1.5 ml centrifuge tube containing 100 μl of Trizol and placed on ice for 30 min, ultrasonicated on ice with a high-intensity sonicator (Sonicator 4000), and centrifuged at 10000 × g (Centrifuge 5424R) for 15 min at 4°C. Finally, the supernatant was collected and analyzed with a BCA assay kit according to the manufacturer's instructions.

Western Blot Identification

Exosomes suspended in PBS were prepared using RIPA lysis buffer (Beyotime Biotechnology, Haimen, China) supplemented with a protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Branford, CT, USA). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Immobilon-Psq, Millipore). The membrane was removed and placed in an incubation cassette containing 5% BSA blocking solution for 2 h at room temperature. The primary antibody was diluted according to the manufacturer's instructions and incubated with the membrane overnight at 4°C on a shaker. Protein band gray value analysis was performed with ImageJ software. The following primary antibodies were used for western blotting: APOH (Abcam), Erk (Cell Signaling Technology), P-Erk (Cell Signaling Technology), p38 (Cell Signaling Technology), P-p38 (Cell Signaling Technology), GAPDH (Cell Signaling Technology), CD63 (System Biosciences), CD81 (System Biosciences), CD9 (System Biosciences), HSP70 (System Biosciences), Calnexin (Cell Signaling Technology) and Grp94 (Cell Signaling Technology).

Quantitative Proteomics Analysis With Total-Protein Tandem Mass Tag (TMT) Labeling

Exosome samples were processed for TMT-labeled quantitative proteomics analysis by Jingjie PTM BioLab (Hangzhou, China). First, the protein sample concentration was determined with a Pierce BCA protein assay kit (Thermo Fisher Scientific). After trypsin digestion, the peptides were desalted with Strata X C18 (Phenomenex) and vacuum freeze-dried. The tryptic peptides were fractionated by high-pH reverse-phase HPLC using an Agilent 300 Extend-C18 column (5 μm particles, 4.6 mm ID, 250 mm length). The peptides were dissolved in 0.1% (v/v) formic acid then loaded onto an EASY-nLC 1000 UPLC system. The peptides were treated with a nanospray ionization source followed by tandem mass spectrometry (MS/MS) in a Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The resulting MS/MS data were processed by the Maxquant search engine (v.1.5.2.8).

Animal Model Establishment and Processing

Female BALB/c nude mice (aged 5 weeks, experimental animal license number m20190601) were randomly divided into four groups and each group was injected with APS-exos (100 μg/ml/each), NC-exos (100 μg/ml/each), N-exos (50 μg/ml/each) or APOH-exos (50 μg/ml/each) *via* the caudal vein once a week for three continuous weeks. Seven days after the final treatment, the mice were mated overnight with male mice. Mating was determined by the appearance of a vaginal plug the following morning. On days 12–14 after mating, all female mice were sacrificed by cervical dislocation. The uteri were removed, and the total numbers of implanted embryos and resorbed embryo-fetal sites were counted. The placental tissue was collected for paraffin sectioning and hematoxylin and eosin (H&E) staining.

Evaluation of Pregnancy Outcomes

The embryo loss rate (ELR) was calculated as the ratio of resorbed embryos/fetuses to the number of all fetuses. The

mean number of embryos implanted (MNEI) was calculated as the ratio of total fetuses to the total number of mice.

Hematoxylin and Eosin Staining

Tissues were fixed with 4% paraformaldehyde for 48 h and processed by conventional procedures. For paraffin-embedded tissue, sections of 3 to 5 μm thickness were cut from paraffin-embedded tissues, mounted on poly-L-lysine-coated slides, deparaffinized in xylene, dehydrated in alcohol, and stained with H&E. Pathological changes in the placenta were observed by H&E staining using electron microscopy (PHILIP CM-120). The vascular condition was evaluated independently by a pathologist (Dr. He).

Statistical Analysis

All data were analyzed with GraphPad Prism 6.0 from GraphPad Software (San Diego, USA). The t-test was used to compare data between two groups, the nonparametric test was applied to non-normally distributed data, Chi-square test and Fisher's exact test were applied for proportions. P-values <0.05 were considered to be statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001; ns = not significant.

RESULTS

Plasma Exosome Isolation and Characterization

We adopted the 2006 International Consensus Statement on the Update of the Classification Standards for APS (17). The clinical features of healthy early pregnancy patients and early pregnancy patients with APS are shown in **Table 1**. There were no significant differences in maternal age (ranged from 25 to 35 years old) or gestational age (gestational age 6–8 weeks) between the two groups. The early pregnancy patients with APS were those with a history of abortion and positive laboratory test results for aPLs. The healthy early pregnancy patients were those with no history of abortion and negative laboratory test results for aPLs.

Purified exosomes were characterized (**Figures 1A–C**). By transmission electron microscopy, circular vesicular particles with a diameter of approximately 100 nm and lipid bilayer membranes were observed (**Figure 1A**). The NTA technique determined particle size distribution and particle concentration of the exosomes. As shown in **Figure 1B**, the particle diameter plot showed a single-peak healthy distribution with peaks at approximately 106.8 nm and 106.7 nm, primarily ranging from 30–150 nm. The particles in the sample were uniformly dispersed. Western blotting identified marker proteins CD9, CD63, CD81 and HSP70 on the serum exosome surface (**Figure 1C**), whereas intracellular proteins Calnexin and Grp94 were not detected. There was no difference in exosomal morphology between NC-exos and APS-exos.

TABLE 1 | The clinical features of healthy early pregnancy patients and early pregnancy patients with antiphospholipid syndrome (APS).

	NC (Mean ± SEM)	APS (Mean ± SEM)
Number	6	6
Maternal age (years)	28.33 ± 1.764	27.83 ± 0.703
Gestation age (days)	42.33 ± 2.951	41.00 ± 2.309
Abortion times	0	2.167 ± 0.167
Antiphospholipid antibody		
Anti-APOH IgM antibody	Negative	52.92 ± 4.565
Anti-APOH IgG antibody	Negative	Negative
Anti- cardiolipin	Negative	Negative
Lupus anticoagulant	Negative	Negative

APS-Exos Cause an Antiphospholipid Syndrome-Like Phenotype in Pregnant Mice

To investigate the effect of APS-exos on embryo resorption, all pregnant mice were sacrificed 12–14 days after plug formation, and embryos and placentas were collected for analysis (**Figure 1D**). Typical embryos and placenta from a mouse treated with NC-exos and APS-exos are shown in **Figures 1E, F**. We found that the resorption rate of the APS-exos group (27.27%) was significantly higher than that of the control group (9.72%), with representative fetal reabsorption observed by the chi-square test (**Figure 1G**, **Table 2**). The MNEI of the APS-exos group was significantly lower than that of the control group (4.4 versus 9.0, respectively, **Figure 1H**). By electron microscopy, the placental angiogenesis of mice treated with APS-exos was decreased, but the difference was not significant (**Figure 2A**). These results indicate that exposure to APS-exos may result in APS-like adverse reproductive outcomes.

APS-Exos Reduced Endothelial Function

To investigate the effect of plasma exosomes on endothelial cells in early pregnancy patients with APS, we performed proliferation, migration, and tube formation assays. APS-exos had no effect on HUVEC proliferation, but significantly inhibited HUVEC migration and impaired tube formation (**Figures 2B–D**).

Quantitative Proteomics Analysis Identified APOH as a Key Target Protein

To determine the difference between APS-exos and NC-exos, we carried out a quantitative proteomics analysis of whole protein TMT markers. A total of 293 proteins were identified from the two groups, including 261 quantified proteins. Values >1.4-fold of the baseline value were chosen as significant upregulation, and <1/1.4-fold was chosen as significant downregulation. Twenty-seven upregulated proteins and nine downregulated proteins were identified. A summary of all the differentially expressed proteins identified in this study are shown in **Figure 3A**. We examined the APOH protein, as it is a major antigen of APS (1, 5). Difference in APOH levels between the two groups were shown by proteomic analysis (**Figure 3B**). Furthermore, the differential protein expression found by TMT analysis was confirmed *via* western blotting, which indicated that APOH was significantly higher in APS-exos than that in NC-exos (**Figure 3C**).

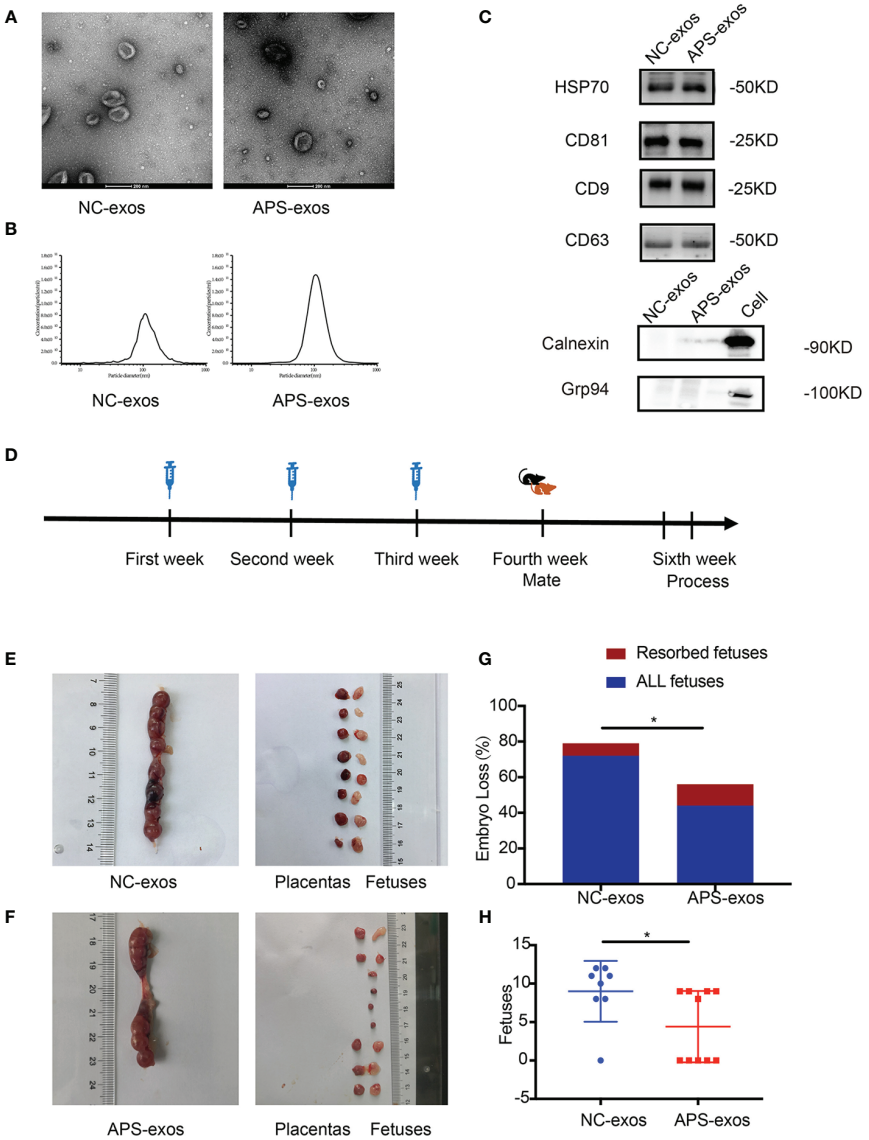

FIGURE 1 | **(A)** Transmission electron microscopy (TEM) images of collected exosomes. Scale bars in the images represent 200 nm. **(B)** Diagram of the particle size distribution obtained by nanoparticle tracking analysis. **(C)** Confirmation of marker proteins in the exosome (exos) preparations. Western blotting of samples for HSP70, CD81, CD9, CD63, Calnexin and Grp94. **(D)** The mice were injected with antiphospholipid syndrome (APS)-exos (100 μg/ml/each) or normal control (NC)-exos (100 μg/ml/each) *via* the caudal vein once a week for 3 continuous weeks. Seven days after the final immunization, the treated female BALB/c mice were mated overnight with male mice. Mating was detected by the appearance of a vaginal plug the following morning. On days 12–14 after mating, all female mice were sacrificed. **(E, F)** Establishment of the abortion model. Typical uterus and embryos from a mouse treated with APS-exos. **(G)** The bar shows the percentage of embryo-fetal loss in the different groups. APS-exos increased the abortion rate of mice (NC-exos, n = 7 and APS-exos, n = 5, *P = 0.038 by chi-square test). **(H)** The scatter plot shows the pregnancy rates in different groups. APS-exos reduced the pregnancy rate. (NC-exos, n=8 and APS-exos, n =10, *P = 0.029 by Mann–Whitney test).

TABLE 2 | Embryo loss rate of antiphospholipid syndrome-exosomes (APS-exos) and control (NC)-exos immunized mice (P = 0.038).

Data analyzed	All fetuses	Normal fetuses	Resorbed fetuses	Embryo Loss Rate	chi-square test	
NS-exos	72	65	7	9.72%		
APS-exos	44	32	12	27.27%	χ^2 = 4.28	P = 0.038
Total	116	97	19			

Gene Ontology (GO) and KEGG Pathway Analysis

The APS-exos- and NC-exos-induced differentially expressed proteins were categorized into GO annotation classes. Based on the above data, we carried out a systematic bioinformatics analysis of proteins containing quantitative information, including protein annotation, functional classification, functional enrichment and clustering analysis based on

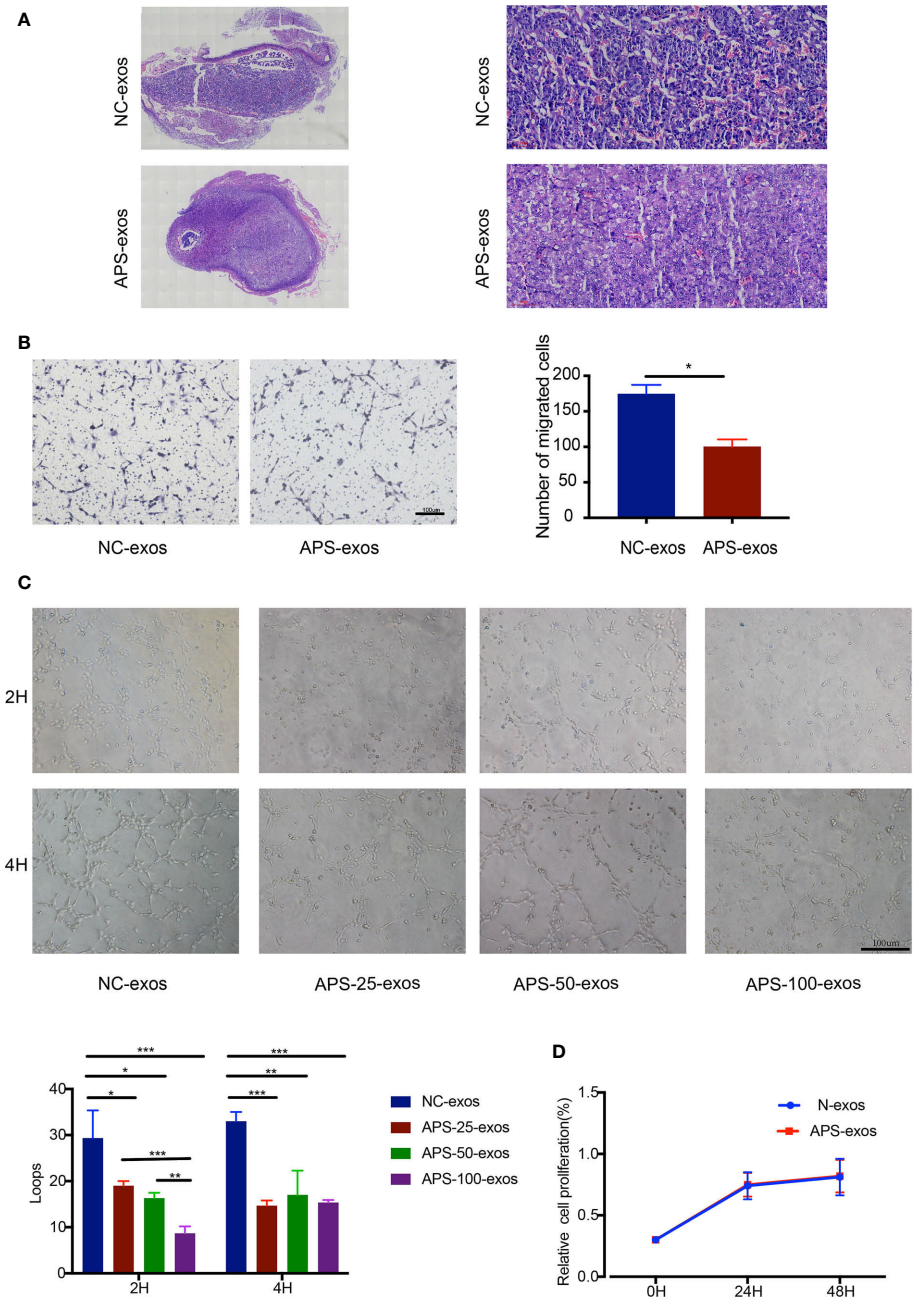

FIGURE 2 | **(A)** Pathological analysis of hematoxylin and eosin (H&E)-stained placental tissue. H&E staining of placental tissue from the antiphospholipid syndrome-exosomes (APS-exos) and normal control (NC)-exos mouse model analyzed by light microscopy (2× and 20×). Placental blood vessels in mice treated with APS-exos compared with those treated with NC-exos. **(B)** After incubation with APS-exos or N-exos (50 μg/ml) for 16 h, human umbilical vein endothelial cell (HUVEC) migration was analyzed. *P = 0.029 by Paired t test. **(C)** After incubation with NC-exos (50 μg/ml) or APS-exos (25 μg/ml, 50 μg/ml, 100 μg/ml) for 2 h and 4 h, angiogenesis of HUVECs was analyzed. *P < 0.05; **P < 0.01; ***P < 0.001 by t test. **(D)** After incubation with APS-exos/NC-exos (50 μg/ml) for 24h and 48h, HUVEC proliferation was analyzed by the cell counting kit-8 assay. *P = 0.183 by Paired t test. All data shown as mean ± S.D.

functional enrichment (**Figures 4A–C**). The upregulated and downregulated proteins are mainly involved in binding, molecular function regulation, catalytic activity, transporter activity and other functions. Differentially expressed proteins were divided into four groups (Q1 to Q4) according to their differential multiples: Q1 (0 < ratio ≤ 1/1.4), Q2 (1/1. < ratio ≤ 1/1.2), Q3 (1.2 < ratio ≤ 1.4) and Q4 (ratio > 1.4). We then enriched the KEGG and protein domains in each Q-group, and a cluster analysis was performed to find the correlation of protein functions with different expression multiples.

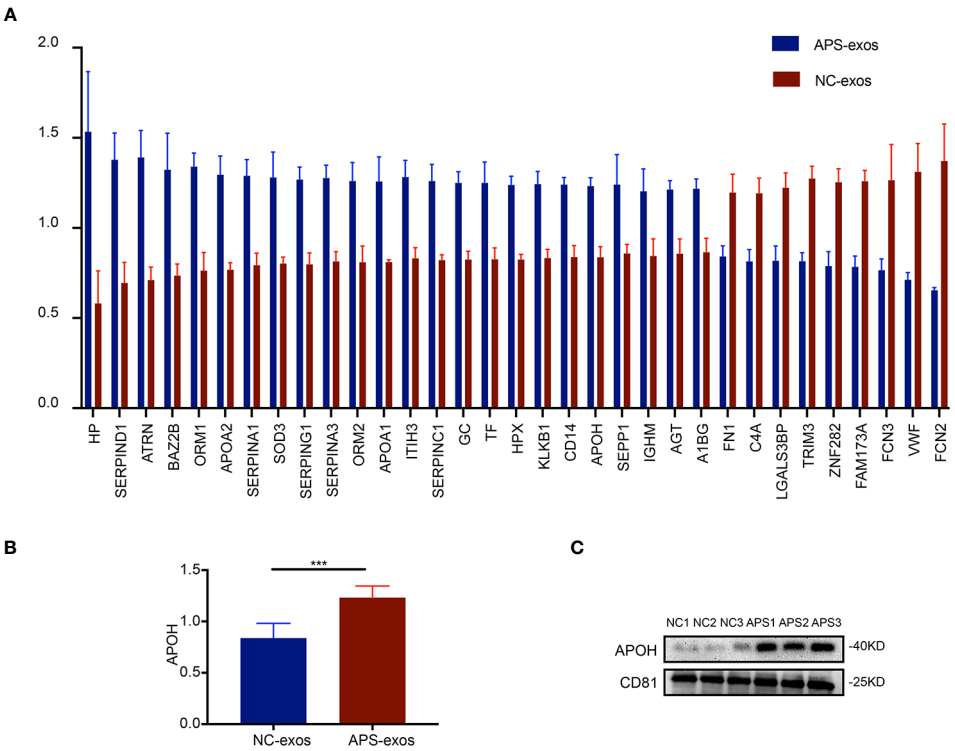

FIGURE 3 | Proteomic analysis results. **(A)** A total of 293 proteins were identified from the two groups. Twenty-seven upregulated proteins and nine downregulated proteins were identified by quantitative proteomics tandem mass tag analysis of antiphospholipid syndrome-exosomes (APS-exos) and normal control (NC)-exos. Mean ± SEM (36 proteins were significantly different, $P < 0.05$ by t test). **(B)** The difference in APOH expression between the two groups shown by proteomic analysis ($P = 0.000$ by t test). **(C)** Clinical validation showed that the level of APOH was significantly higher in APS-exos than that in NC-exos. "***" means $P = 0.000$ by t test.

APOH-Exos Caused an Antiphospholipid Syndrome-Like Phenotype in Pregnant Mice

To investigate the effect of APOH on endothelial cells, we constructed APOH-overexpressing lentiviral vectors and transduced the APOH lentivirus into 293T cells to enhance APOH expression (APOH-293T). Compared with the NC vector (N-293T), the APOH vector increased the expression of APOH protein in 293T cells and the secreted exosomes (**Figures 5A, B**).

To identify the effects of APOH-exos on embryo resorption, all pregnant mice were sacrificed 12–14 days after plug formation, and the embryos and placenta were sampled for further analysis (**Figures 5C, D**). We found that the embryo-fetal resorption rate for the APOH-exos group (27.78%) was significantly higher than that of the control group (4.91%); representative reabsorption is shown in **Figure 5E** and **Table 3**. There was no significant difference in MNEI levels between the APOH-exos and control groups (**Figure 5F**). By electron microscopy, placental angiogenesis of mice treated with APOH-exos was not significantly different compared with control mice (**Figure 5G**).

Inhibition of HUVEC Migration and Tube Formation by APOH-Exos

To determine the relationship between APOH-exos and HUVEC function, we performed proliferation, migration, and tube formation assays. APOH-exos had no effect on proliferation but significantly inhibited HUVEC migration and reduced tube formation (**Figures 6A–C**).

APOH-Exos Inhibited the Phosphorylation of Erk1/2

To further investigate the mechanism of APOH-exos actions, APOH-exos were observed by laser scanning confocal microscopy (**Figure 6D**). The binding and endocytosis of APOH-exos were observed in HUVECs. After 16 h of incubation of HUVECs with APOH-exos, the level of phospho-extracellular signal-regulated kinase 1/2 (P-Erk1/2, **Figure 6E**) was decreased, whereas the levels of Erk1/2, p38 and phospho-p38 (P-p38) were the same. APOH may inhibit HUVEC angiogenesis through the P-Erk signaling pathway.

DISCUSSION

Exosomes carrying proteins and other agents play a role in intercellular communication. There is increasing evidence showing exosomes play a critical role in pregnancy (18, 19). APS is a unique multiple-system disorder that leads to thrombosis and/or pregnancy complications. Here, we provide evidence that exosomes containing APOH protein from early

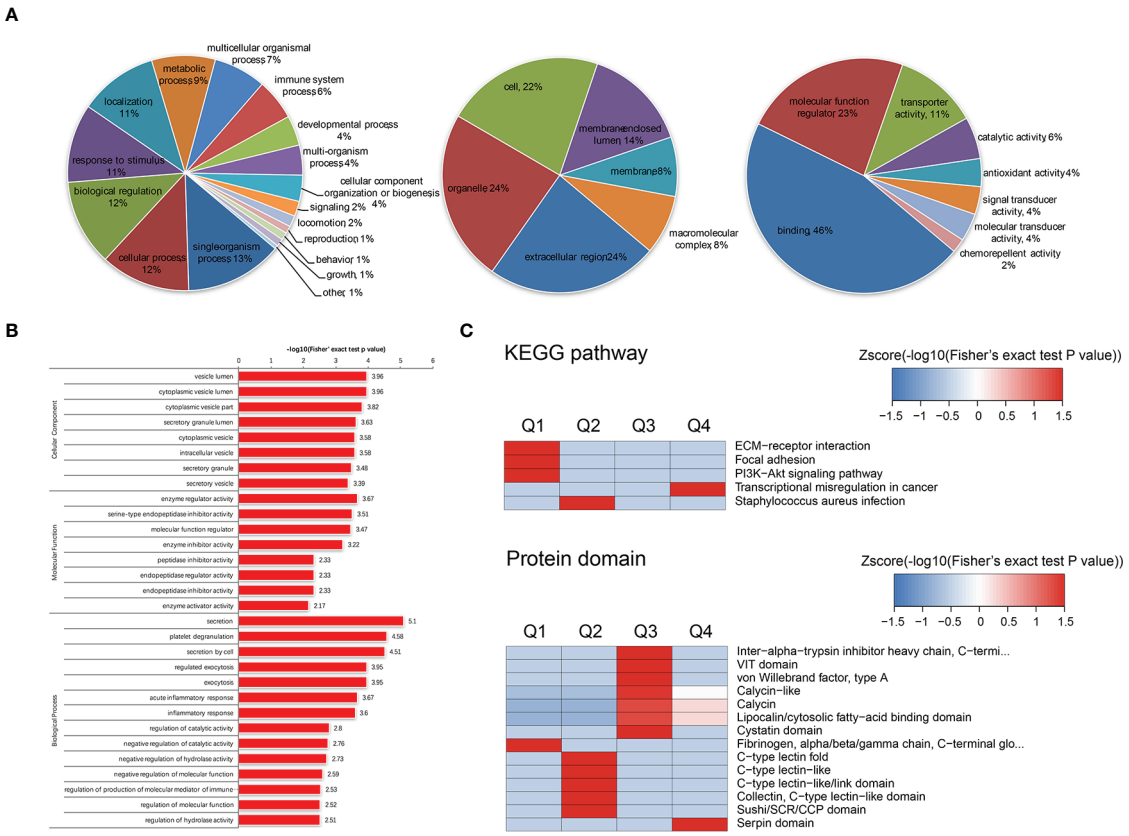

FIGURE 4 | **(A)** Gene Ontology (GO) annotation was derived from the UniProt-GOA database. GO enrichment analysis of upregulated proteins in the biological process (left), cellular component (mid), and molecular function (right) categories. **(B)** We enriched and analyzed the entries in GO annotations except for the first three categories (biological process, cellular component and molecular function). **(C)** The clustering method is based on the p value of Fisher's exact test obtained from enrichment analysis. The hierarchical clustering method was used to gather relevant functions in different groups and draw them as a heat map. The horizontal of the thermogram represents the enrichment test results of different groups, and the vertical represents the description of differential enrichment related functions (KEGG pathway, protein domain). The different proteins and the color blocks corresponding to the functional description in different groups indicate the degree of enrichment. Red indicates a strong degree of enrichment, while blue indicates a weak enrichment. KEGG pathways with significant enrichment of differentially expressed proteins [antiphospholipid syndrome-exosomes (APS-exos) versus normal control-exos) (above). Cluster analysis heat map based on protein domain enrichment (below).

pregnancy patients with APS can induce vascular endothelial dysfunction and facilitate adverse reproductive events.

Angiogenesis is a pivotal process in embryo development and tissue growth, but also acts as a key player in many pathological conditions. Angiogenesis includes several steps characterized by endothelial cell function, such as migration and lumen formation (20). APLs in APS can activate endothelial cells and exosome release. Studies have shown a significant increase in the level of exosomes in patients with APS, but the relationship between exosomes and thrombosis and obstetric events has not been confirmed (16). In this study, we revealed that the exosomes of patients with APS can cause adverse reproductive events in mice. We found that APS-exos increased the abortion rate and reduced the successful pregnancy rate of mice. At present, the reason for the APS-exos-induced reduction of successful pregnancy in mice remains undetermined. We found that the abortion rate was increased in the APS-exos mouse model, and we also confirmed that exosomes from patients with APS induced HUVECs dysfunction. Therefore, we hypothesized that some proteins in exosomes of APS patients lead to an increased abortion rate by inhibiting angiogenesis. To explore the exosome proteins involved in APS-like actions, we compared proteomic profiles using high-resolution TMT technology (21).

The APOH protein levels found by protein mass spectrometry attracted our attention. APOH is an important antigen of APS, and the antigen-antibody complex of antiphospholipid antibodies and APOH increases the affinity for negatively charged phospholipids on the cell surface, thereby activating endothelial cells, mononuclear cells and platelets (1, 5, 22, 23). Past work showed angiogenesis was significantly higher in APOH gene-deficient mice compared with healthy mice (24). Treatment with APOH has been shown to induce experimental antiphospholipid syndrome in naive mice and can induce APS-like characteristics in mice (23).

In the current study, 293T cells overexpressing APOH by lentivirus transduction produced exosomes that also contained

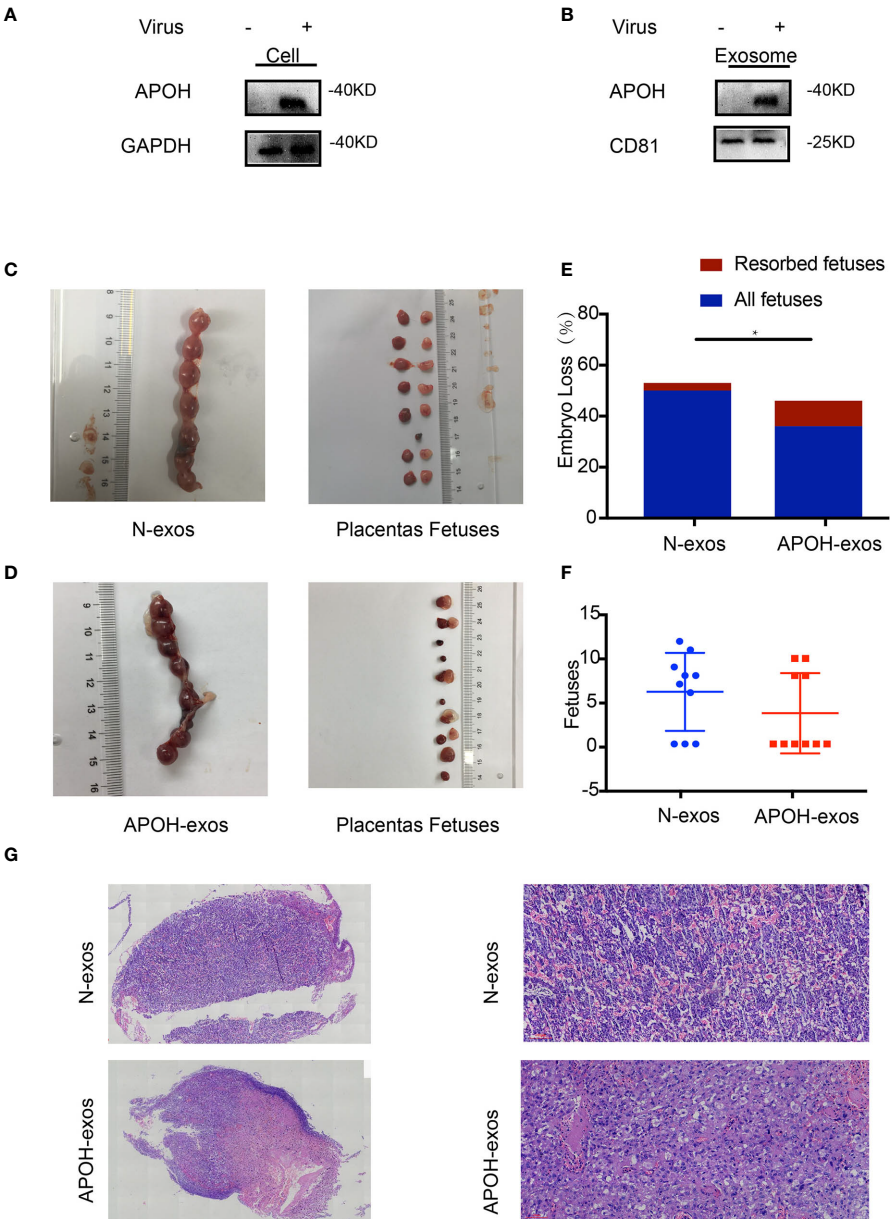

FIGURE 5 | **(A, B)** Apolipoprotein H (APOH) lentivirus was transduced into 293T cells to enhance APOH expression. APOH overexpression and control (NC) lentiviral vectors were constructed. The cells and exosomes [N-exosomes (exos) and APOH-exos] were examined by western blotting. **(C, D)** Establishment of the abortion model is shown in **Figure 1D**. Typical embryos and uterus from a mouse treated with APOH-exos and NC-exos (50ug/ml/each). **(E)** APOH-exos increased the abortion rates of mice (NC-exos, n = 7 and APOH-exos, n = 4; *P = 0.012 by Fisher's exact test). **(F)** No significant difference in pregnancy rate between the two groups. (NC-exos, n =10 and APS-exos, n =10; *P = 0.319 by Mann–Whitney test). **(G)** Pathological analysis of hematoxylin and eosin-stained placental tissue from the APOH-exos mouse model by light microscopy (2× and 20×). The placental blood vessels and the vascular thrombus in mice treated with APOH-exos compared with those in mice treated with N-exos.

TABLE 3 | Embryo loss rate of Apolipoprotein H-exosome (APOH-exos) and control (N)-exos immunized mice (P = 0.012).

Data analyzed	All fetuses	Normal fetuses	Resorbed fetuses	Embryo Loss Rate	Fisher's exact test
N-exos	50	47	3	4.91%	P = 0.012
APOH-exos	36	26	10	27.78%	
Total	86	73	13		

high levels of APOH. We found that the abortion rate increased in APOH-exos-treated mice. In vitro experiments showed that APOH-exos can also induce endothelial dysfunction and affect angiogenesis. Previous studies have shown that aPLs from patients with APS activated cells *via* the p38 and Erk pathways (25–27). We further explored the underlying mechanism and found Erk1/2 phosphorylation was significantly inhibited in the

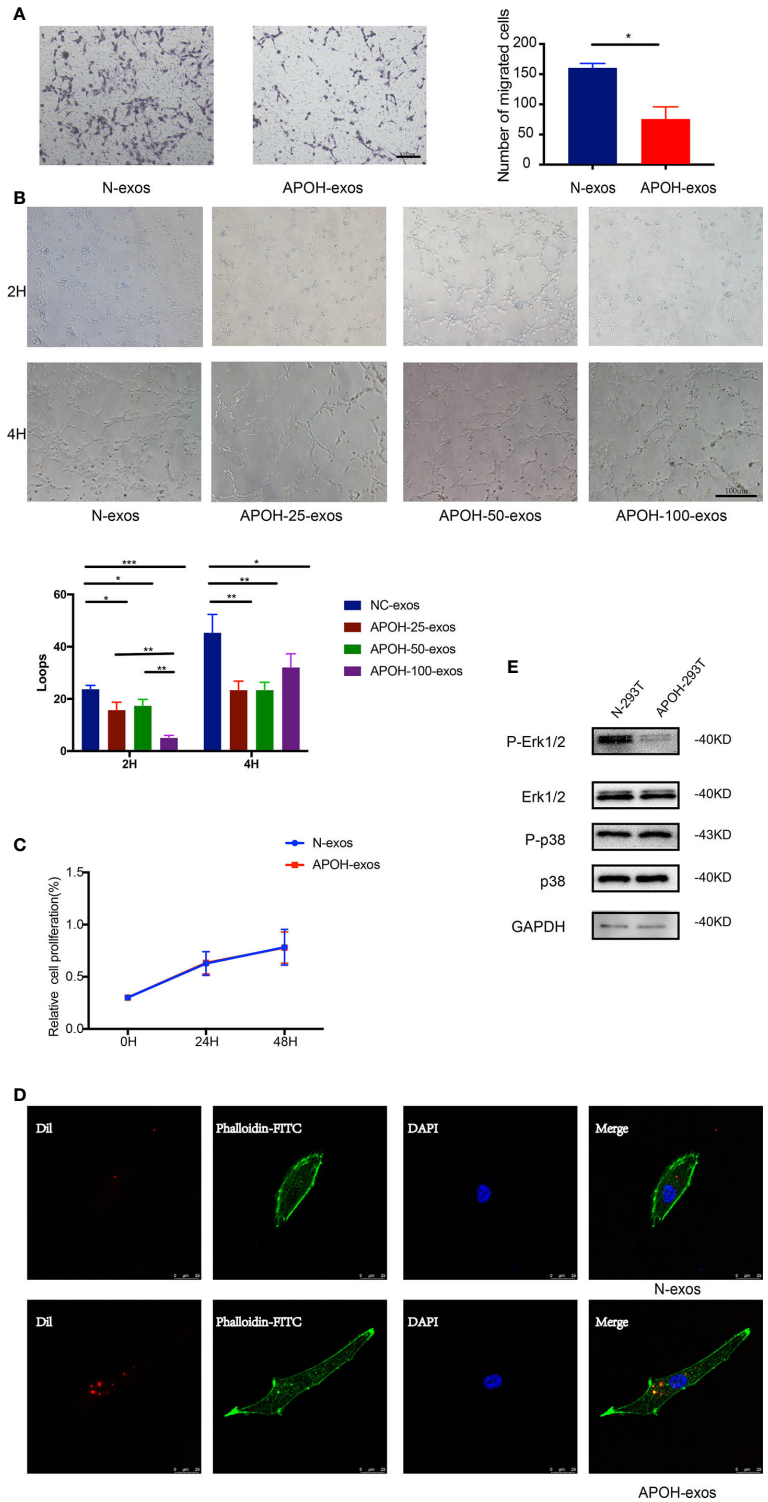

FIGURE 6 | **(A)** After incubation with 25 μg/ml APOH-exosome (exos) or 25 μg/ml control (N)-exos for 16 h, human umbilical vein endothelial cell (HUVEC) migration was analyzed. Mean ± SD (*P = 0.014 by Paired t test). **(B)** After incubation with N-exos (50 μg/ml) or APOH-exos (25 μg/ml, 50 μg/ml, 100 μg/ml) for 2 h and 4 h, HUVEC angiogenesis was analyzed. Mean ± SD (*P < 0.05; **P < 0.01; ***P < 0.001 by t test). **(C)** After incubation with APOH-exos/N-exos (25 μg/ml) for 24h and 48h, HUVEC proliferation was analyzed by the cell counting kit-8 assay. Mean ± SD (*P = 0.183 by Paired t test). **(D)** The binding and endocytosis of APOH-exos/N-exos in HUVECs was observed by laser scanning confocal microscopy. **(E)** After 16 h of incubation of HUVECs with APOH-exos, the levels of Erk 1/2, P-Erk 1/2, p38 and P-p38 were determined by western blotting.

APOH-exos group compared with the control group, whereas Erk1/2, p38 and P-p38 levels were equivalent in both groups, suggesting that the P-Erk signaling pathway was inhibited by APOH. P-Erk is a member of mitogen-activated protein kinase (MAPK) signaling pathway, and plays an important role in cell growth, migration and angiogenesis (25–33). APOH-exos may inhibit P-Erk1/2, then inhibit cell migration and tube formation, which means the inhibit of angiogenesis. Our data suggest that APOH protein in exosomes from APS patients might inhibit angiogenesis through the P-Erk pathway, resulting in an increased rate of abortion. Importantly, APOH-exos can be directly transferred into HUVECs and inhibit endothelial cell migration and tube formation through the suppression of P-Erk, rather than activating the pathway by binding the antiphospholipid antibody and antigen to the surface of the phospholipid (34).

In conclusion, we propose that APS-exos may be a key factor in the pathogenesis of APS and that APOH may be a key protein that impairs vascular biological function. Additional studies are required to determine the mechanisms by which exosomal APOH induces vascular dysfunction and to investigate the potential use of specific inhibitors to block relevant pathways to treat APS.

DATA AVAILABILITY STATEMENT

All the data and techniques within the article are available from the corresponding authors upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Shanghai First Maternal and Infant Hospital affiliated with Tongji University (KS18128). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of East China Normal University (m20190601). Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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