Journal of Nanobiotechnology

REVIEW

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Extracellular vesicles: a new frontier in diagnosing and treating graft-versus-host disease after allogeneic hematopoietic cell transplantation

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Abstract

Graft-versus-host disease (GvHD) is a prevalent complication following allogeneic hematopoietic stem cell transplantation (HSCT) and is characterized by relatively high morbidity and mortality rates. GvHD can result in extensive systemic damage in patients following allogeneic HSCT (allo-HSCT), with the skin, gastrointestinal tract, and liver frequently being the primary target organs affected. The severe manifestations of acute intestinal GvHD often indicate a poor prognosis for patients after allo-HSCT. Endoscopy and histopathological evaluation remain employed to diagnose GvHD, and auxiliary examinations exclude differential diagnoses. Currently, reliable serum biomarkers for the diagnosis and differential diagnosis of GvHD are scarce. As an essential part of standard transplant protocols, early application of immunosuppressive drugs effectively prevents GvHD. Among them, steroids represent first-line therapeutic agents, and the JAK2 inhibitor ruxolitinib represents the second-line therapeutic agent. Currently, no efficacious treatment modality exists for steroid-resistant aGvHD. Therefore, the diagnosis and treatment of GvHD still face significant medical demands. Extracellular vesicles (EVs) are nanometer to micrometer-scale biomembrane vesicles containing various bioactive components, such as proteins, nucleotides, and metabolites. Distinctive changes in serum-derived EV components occur in patients after allo-HSCT; Hence, EVs are expected to be potential biomarkers for diagnosing and treating GvHD. Furthermore, cell-free therapeutics characterized by EVs derived from mesenchymal stem cells (MSCs) have manifested remarkable therapeutic efficacy in preclinical models and preclinical trials of GvHD. Customized engineered EVs with fewer toxic and side effects for the combined treatment of GvHD hold broad prospects for clinical translation. This review article examines the

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potential value of translating EVs into clinical applications for the diagnosis and treatment of GvHD. It summarizes the latest advancements and prospects of engineered EVs applying GvHD.

Keywords Graft-versus-host disease, Extracellular vesicles, Biomarkers, Mesenchymal stem cells, Therapeutics

Introduction

Graft-versus-host disease (GvHD) is a serious complication of allo-HSCT [1]. According to the latest survey statistics, the cumulative incidence of acute GvHD (aGvHD) reported by each center within 100 days was 62%. After verification by the endpoint review committee, the incidence rate has been revised to 49%. 40% of all patients after allo-HSCT experience aGvHD (grades II-IV), with 19% having severe aGvHD (grades III-IV) in patients who received a transplant during the period 1990–1995. The incidence of aGvHD is declining over time. Among patients who received transplants between 2011 and 2015, the incidence of aGvHD in grades II-IV was 28%, and the incidence of grades III-IV aGvHD was 11% [2–4]. In recent decades, the primary clinical treatment of GvHD still relies on inducing overall immunosuppression (5–6). However, due to the need for more accuracy of this method, it leads to a high non-recurrence mortality rate secondary to infection. Also, it increases the risk of resistance to treatment and relapse of blood cancer [6]. Therefore, exploring new prevention and treatment methods in the clinic is urgent.

Extracellular vesicles (EVs) constitute a group of membranous vesicles enclosed by a lipid bilayer ranging from the nano- to micrometer scale (7–8). Many recent studies have indicated that EVs are vital in the genesis, progression, diagnosis, treatment, and prognosis of immune disorders [9–12]. EVs can be discharged into the extracellular milieu via the active secretion of viable cells or the exfoliation of dead cells, thereby being ubiquitously present in diverse body fluids and cell supernatants [13]. Because of their contents, various proteins, nucleotides, lipids, and metabolites are often used as carriers for various diseases [14]. Furthermore, these components can precisely reflect the characteristics of the source cells, so EVs are also valuable biomarkers for disease diagnosis [13, 15]. Studies have shown that the components of EVs in the serum of patients after allo-HSCT are unique. Hence, EVs have potential value in early diagnosis and monitoring of the prognosis of GvHD [16–18]. Furthermore, clinical and preclinical studies have shown that EVs, as an essential component of paracellular secretion, have biological functions similar to those of parental cells (19–20), such as immune regulation, proliferation promotion and inhibition of apoptosis, and anti-inflammatory and antioxidant functions. Mesenchymal Stromal/Stem Cells (MSCs) are pluripotent stem cells with self-renewal and multi-differentiation potential [21–23]. MSCs are a potential treatment for GvHD due to their multiple biological properties, such as immune regulation [24]. In recent years, it has been widely documented that EVs derived from mesenchymal stem cells (MSCs) are capable of evading numerous side effects and substituting for MSCs in exerting therapeutic functions, concurrently significantly enhancing the clinical manifestations and mortality of GvHD [25–27]. In addition, researchers have also found that EVs can serve as an excellent delivery carrier [28], and the surface and interior of its lipid bilayer can be personalized and loaded with some tissue-targeted molecules or enhanced therapeutic drugs to improve its efficacy in intervening GvHD [29].

Literature reviews on EVs and GvHD reported thus far have mainly emphasized the role and mechanism of EVs in the pathogenesis and progression of GvHD. In contrast, our review will concentrate primarily on the clinical diagnostic value of EVs in diagnosing GvHD and the translational application value of MSC-EVs in treating GvHD. More specifically, this review will discuss the occurrence and development of GvHD from multiple standpoints, including the pathogenesis, classification, and the latest therapeutic progress of GvHD, and review the progress of applying EV-based diagnosis and treatment in GvHD, as well as future clinical application prospects.

Classification and pathogenesis of GvHD

Allo-HSCT is an efficacious modality for managing diverse malignant hematological diseases, including leukemia, lymphoma, myelodysplastic syndrome, aplastic anemia, and multiple myeloma [30–32]. In 1990, the results of the first survey conducted by the European Bone Marrow Transplant Group indicated that a total of 143 centers reported 4,234 hematopoietic cell transplants (HCT). As of 2019, a total of 700 centers from 51 countries reported that the number of HCT in Europe and its partner countries reached 48,512, among which 19,798 cases (41%) were allogeneic transplants and 28,714 cases (59%) were autologous transplants (33–34). In 2018, over 22,000 cases of HCT were reported in the United States [35]. Currently, haploid HSCT (haplo-HSCT) is widely used worldwide, and with the successful application of haplo-HSCT, the number of hematopoietic HSCT cases has increased rapidly. Over the past three decades, the scale of haplo-HSCT in Europe has witnessed a remarkable increase. By 2019, the quantity of haplo-HSCT had nearly multiplied by eight [34, 36]. Data from the Chinese Hematopoietic Stem Cell Transplantation Registry

showed that in 2019, almost 10,000 cases of allo-HSCT were performed in 140 medical institutions in China, among which haplo-HSCT accounted for 60% [37–38]. Although the therapeutic efficacy of allo-HSCT in the treatment of malignant hematological diseases has been continuously improved, the frequent occurrence of complications such as GvHD, infections, toxic reactions, and relapses still constitutes significant factors restricting the success of transplantation.

GvHD is caused by the active attack of T lymphocytes on allogeneic donor grafts against alloantigens in recipients after allo-HSCT, primarily targeting the skin, gastrointestinal tract, and liver, manifesting as skin congestion, maculopapular rash, and intractable diarrhea (4–5). Based on the timing of the GvHD after allo-HSCT, GvHD is mainly divided into two forms: acute GvHD (aGvHD) that occurs within 100 days and chronic GvHD (cGvHD) within more than 100 days. In 2005, the National Institutes of Health (NIH) of the United States refined this classification and distinguished GvHD based on disease characteristics [39]. aGvHD mainly affects the skin, liver, and gastrointestinal tract, while cGvHD may affect any body organ, and its diagnosis is not time-limited [40]. The pathophysiological process of aGvHD is mainly divided into three stages: the initiation phase, the phase of T cell activation, and the effector phase [4]. At the initiation stage, the intervention of chemotherapy and/or radiotherapy induces the release of danger signals in the host tissues, such as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), which further activates the host antigen-presenting cells (APC). APC mainly includes professional APC (dendritic cells) and non-professional APC (epithelial and mesenchymal cells) [41]. APC, beyond merely presenting self-antigens and allogeneic antigens to invigorate pathogenic T cells, orchestrates the development of GvHD by penetrating tissues and organs, secreting a cascade of inflammatory cytokines, and generating both autoantibodies and alloantibodies via an array of intricate mechanisms [42]. In contrast to aGvHD, donor and host APC can initiate CD4-mediated cGvHD [43]. Therefore, blocking donor T cells from recognizing the recipient's alloantigen is a promising strategy for preventing GvHD [44]. In the subsequent stage of T cell activation, the host APC activates the allogeneic donor $CD4^+$ and $CD8^+$ T cells. Subsequently, in the effector stage, effector T cells and pro-inflammatory cytokines lead to the damage or apoptosis of tissues such as the gastrointestinal tract, skin, and liver, thereby triggering the emergence of aGvHD symptoms (4–5). Compared with aGvHD, the pathogenesis of cGvHD is more complex. It is usually divided into three stages: acute inflammation, chronic inflammatory immune dysregulation, and abnormal tissue repair leading to continuous fibrosis (45–46). During the first stage, the inflammatory signals initiated by the injury of the host tissue stimulate the activation of donor alloreactive T cells, thereby causing damage or apoptosis of the host target cells. In the second stage, the peripheral tolerance of regulatory T cells and B cells is lost, concurrently, self-reactive and alloreactive T cell populations emerge. These factors contribute to the persistence of the T17 cell-mediated inflammatory response. In the third stage, activated macrophages stimulate the generation of transforming growth factor-β (TGF-β) and platelet-derived growth factor-α (PDGF-α), which further impairs the function of regulatory T cells and intensifies the inflammation [47]. The abnormal tissue repair process results in continuous fibrosis, and patients may present with scleroderma or obliterative bronchiolitis syndrome. Moreover, the donor B cell's steady state and tolerance mechanism are disrupted, leading to decreased memory function. Donor T follicular helper cells (Tfh) have been verified to proliferate in secondary lymphoid organs and, by secreting cytokines such as IL-21 and B cell activating factors, facilitate the survival and proliferation of donor B cells and their differentiation into plasma cells that generate abnormal anti-host immunoglobulins [48]. B-cell lymphocytopenia and humoral immunodeficiency are prominent features of cGvHD, which may result in the body not producing sufficient antibodies or antibody deficiency, resulting in increased susceptibility to infection by pathogens [49]. Therefore, preventive strategies that block the development of pathogenic B cells or inhibit Tfh cells may better prevent cGvHD (Fig. 1) [46, 50]. In addition to T and B cells, macrophages, NK cells, and other immune microenvironment cells also play a role in aGvHD and cGvHD [49].

Classification, biosynthesis, release, and uptake of EVs

EVs are membrane vesicles of nanoscale to microscale size that are actively released or shrunk by cells into the extracellular environment. According to EVs' biological process, diameter size, and biophysical and chemical properties, the International Society for Extracellular Vesicles has further classified EVs [8, 51]. According to the diameter, they are divided into apoptotic bodies (ABs), microparticles, microvesicles (MVs), nanovesicles, and nanoparticles; according to the different cellular and tissue sources, they are divided into prostasomes, dexosomes, oncosomes, synaptic vesicles, cardiosomes, and vexosomes; and according to function, they are divided into matrix vesicles. According to the extracellular secretion characteristics, they are divided into exosomes, exosome-like vesicles, and ectosomes. Exosomes are vesicles formed by the fusion of multivesicular bodies (MVBs) and the plasma membrane. Microvesicles are vesicles formed by the plasma membrane directly to the outgoing

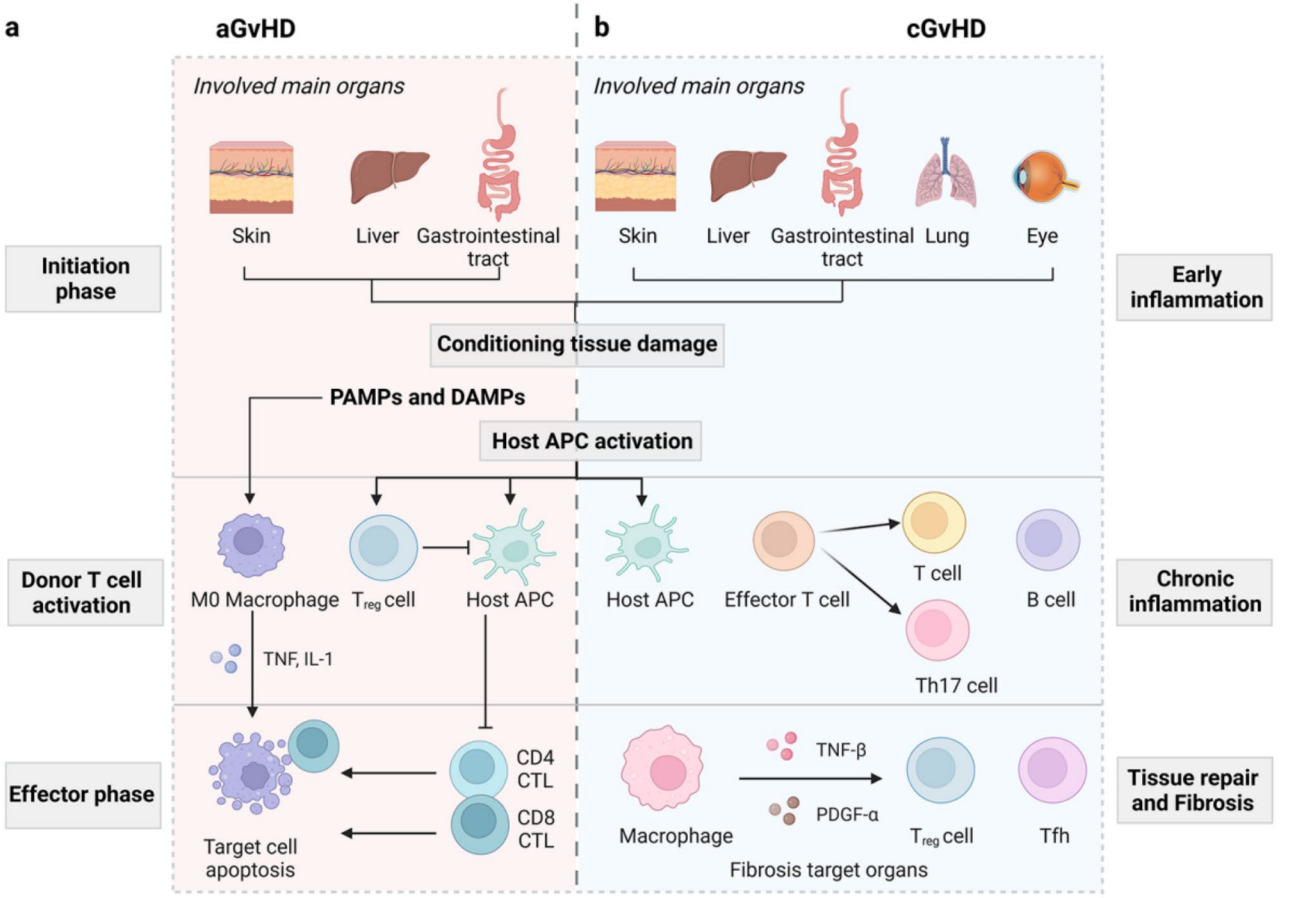

Fig. 1 Classification and pathogenesis of GvHD

GvHD is a multi-system injury disease caused by the donor's immune cells (mainly T cells), which recognize the antigen of the recipient tissue and launch an immune attack after organ transplantation. Based on the timing of the GvHD after allo-HSCT, GvHD is mainly divided into two forms: GvHD, which occurs within 100 days, and cGvHD, which occurs within more than 100 days. GvHD, Graft-versus-host disease; aGvHD, Acute GvHD; cGvHD, Chronic GvHD; IFN-γ, Interferon-gamma; TNF-α, Tumor necrosis factor-alpha; TNF-β, Tumor necrosis factor-beta; Th2 cell, Helper T cell 2; Th17 cell, Helper T cell 17; B cell, B lymphocyte. Figure 1 was created with BioRender software (https://app.biorender.com/)

bud, and apoptotic bodies are vesicles released by apoptosis [52]. Current research has mainly focused on the following three categories: apoptotic bodies, microvesicles, and exosomes. After their initial discovery, EVs were considered to be cell fragments. EVs transfer bioactive proteins or genetic material carried by their internal or surface from donor cells to recipient cells through direct membrane fusion, ligand-mediated interaction, a variety of cell endocytic pathways, and other mechanisms, thus playing a variety of biological functions (Fig. 2) [7, 52]. Tissues and cells in a diseased state specifically express specific ligand molecules. Based on this physiological and pathological process, researchers have designed various specific targeting short peptide molecules such as RVG (Rabies virus glycoprotein) peptide, RGD (Arg-Gly-Asp) peptide, and Kim-1 (Kidney injury molecule-1) peptide to modify the surface of EV vesicles, thereby achieving precise drug delivery [28, 53–55]. In addition, the ligand of CXCR4 (C-X-C chemokine receptor type 4), namely SDF-1/CXCL12 (Stromal cell-derived factor-1), is highly expressed in the inflammatory site. Research indicates that EVs overexpressing CXCR4 can specifically be home to the inflammatory regions, which offers novel ideas for treating diseases such as ulcerative colitis, tumors, osteoporosis, and periodontitis [56–59]. Recently, Stanford et al. [60]. have developed bioengineered multifunctional EVs for targeted delivery of biological agents to T cells. These EVs can specifically target T cells and actively enrich target cargo molecules. Furthermore, this technology significantly improves the efficiency of uptake and fusion of engineered EVs by receptor cells. In contrast to traditional engineering approaches, this novel method does not require the isolation of EVs synthesized by parental cells in vitro. Instead, it enables in situ engineering of parental cells in vivo through a one-step process, allowing the biologically synthesized multifunctional EVs

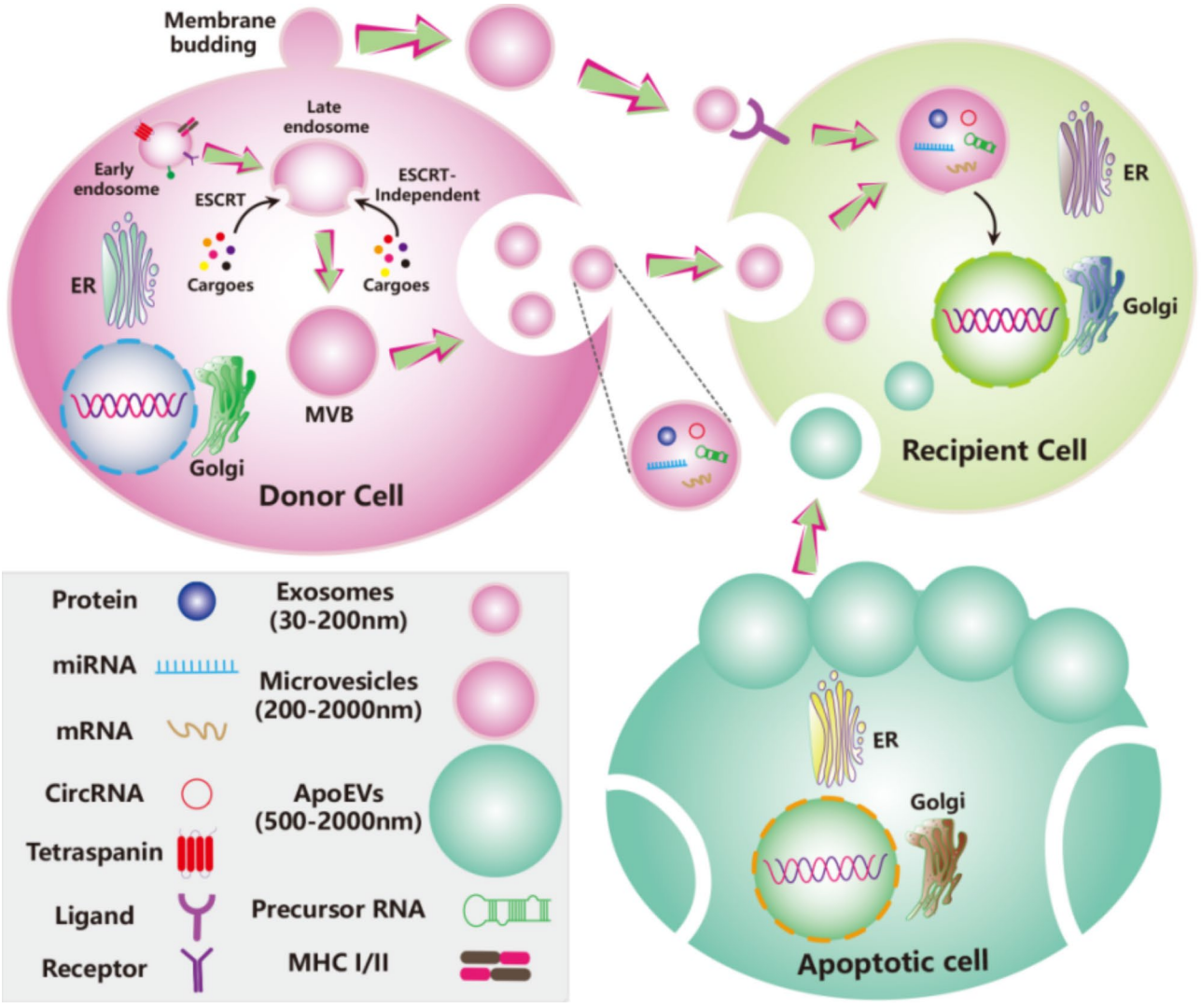

Fig. 2 Biogenesis and function of EVs
EVs are small membrane-bound vesicles actively secreted or released by living cells into the extracellular environment. They have a lipid bilayer membrane surrounding them, are relatively small, and typically range in diameter from 30 to 2000 nm. Small EVs are synthesized and secreted by the parent cell via ESCRT-dependent or -independent pathways and are called exosomes. Meanwhile, the parent cell releases larger EVs, called microvesicles, through budding. ApoEVs, on the other hand, are membrane-bound vesicles that are passively shed by dying cells and are relatively larger, typically ranging from 500 to 2000 nanometers in diameter. ApoEVs originate from the detachment of apoptotic cells. The molecular mechanisms used by receptor cells to take in different-sized EVs include membrane fusion, ligand-mediated, and phagocytosis, among others. ER, Endoplasmic reticulum; Golgi, Golgi apparatus; ESCRT, Endosomal sorting complex required for transport; MVBs, Multivesicular bodies

to be directionally transported via the blood circulation to target cells and directly exert their functions.

After in-depth studies over the past decades, various biological effects of EVs have gradually emerged, including mediating intercellular signaling, antigen presentation, immune regulation, and promotion of angiogenesis (61–62). EVs, particularly those derived from MSCs, exert a favorable therapeutic impact in various refractory diseases, including diabetes, systemic lupus erythematosus, spinal cord injury, malignant hematological disorders, and the associated GvHD [27]. In addition, EVs also have good biocompatibility, low immunogenicity and toxicity, biodegradability, and other advantages, and have gradually become crucial in nanomedicine research [28, 55]. Engineered EVs also bring new hope for personalized and accurate treatment of various diseases [7, 55, 63]. The lipid bilayer of EVs is wrapped with many proteins, nucleotides, lipids, and metabolites, which are used as biomarkers for diagnosing and treating disease. Various cells secrete and release EVs in different disease conditions, carrying proteins, nucleic acids, and lipids. The content carried by EVs serves as a reflection of the cellular state of the parent cell at the time of secretion, and these components undergo dynamic adjustments in

response to changes in the physiological state and environment of the parent cell during EV secretion. Consequently, by analyzing these EVs, we can accurately and precisely reflect alterations in the pathophysiological state of their originating parent cells. Furthermore, given that EVs have specificity and stability of cell types and can be obtained from body fluids, various components within EVs can become suitable biomarkers for early diagnosis, treatment, prognosis, and other diseases. EVs as biomarkers are essential for individualized disease diagnosis and monitoring [64].

The diagnostic value of EVs in aGvHD and cGvHD

aGvHD and cGvHD represent some of the most vital long-term complications ensuing from allo-HSCT and constitute the prime cause of non-relapse mortality [47]. aGvHD and cGvHD not only significantly reduce long-term survival and quality of life for patients post-transplantation but have also been associated with a variety of other comorbidities. Although current research has improved our understanding of the pathogenesis of aGvHD and cGvHD, reliable biomarkers for predicting the occurrence of aGvHD and cGvHD have yet to be determined. Since 30–70% of patients develop GvHD after allo-HSCT [40], it is urgent to identify appropriate early diagnosis and prognostic markers for the early detection and intervention of this post-transplant complication. A diverse array of biomarkers for GvHD, encompassing genetic, plasma, cellular, and microbiome-related markers, is being progressively explored [65]. Within the clinical realm, dependable serum biomarkers for the diagnosis and differential diagnosis of aGvHD and cGvHD remain scarce. Endoscopy, other supplementary examinations, and histopathological assessment still constitute the predominant methods for the diagnosis and differential diagnosis of aGvHD. Liquid biopsy is a non-invasive blood testing approach that analyzes and diagnoses diseases like cancer by monitoring biomarkers such as free circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), exosomes, and circulating RNA that are released into the blood by tumors or metastatic foci [66]. With the rapid evolution of liquid biopsy technology, the screening of biomarkers for the diagnosis and prognosis of GvHD in blood, urine, and other biological fluids holds considerable value. As an essential component of liquid biopsy, EVs are expected to be an attractive biomarker for the diagnosis and prognosis of GvHD due to their high number and easy capture.

The source or quantity of circulating EVs and their diagnostic application in GvHD

The incidence and mortality of cardiovascular events in allo-HSCT survivors are significantly increased, with endothelial dysfunction and thrombosis risk being the main contributors. Therefore, early monitoring of vascular injury and prothrombotic activity in allo-HSCT survivors may be an essential strategy for preventing and treating cardiovascular diseases. In establishing and developing plant resistance to viral host, the number, membrane antigen subgroups, and contents carried by EVs undergo significant changes. Studies have shown that circulating EVs derived from allo-HSCT survivors may be an evaluation index of endothelial injury and procoagulant activity [67]. The study recruited 45 survivors post-allo-HSCT and 45 controls. Most patients who underwent allo-HSCT experienced aGvHD (44%) and/or cGvHD (66%). The authors found that allo-HSCT survivors had significantly increased levels of platelet and erythrocyte-derived EVs compared with the control group [67]. Furthermore, among allo-HSCT survivors with a history of thrombotic microangiopathy, erythrocyte-derived MVs are markedly elevated. Several studies have shown that the number of EVs in the circulating blood of patients with GvHD is significantly increased, and the increase in the secretion of EVs is significantly associated with the risk of aGvHD (16–17). Carneiro et al. [16]. found that in patients with circulating EVs in plasma, high levels of total EVs (TEVs) and red blood cell-derived EVs (EryEVs) counts strongly correlated with the occurrence of aGvHD. In contrast, platelet-derived EVs (PEVs) were not associated with the risk of aGvHD. Some studies have shown that EVs derived from red blood cells, platelets, or vascular endothelial cells may be a new class of biomarkers. The proportion and functional alterations of T cell subsets are paramount for the occurrence and progression of GvHD. Moreover, T cells released EVs at a constant low level, which suddenly increased after activation. Hence, detecting the variations of EV subpopulations derived from T cells might offer a more direct and efficient means for monitoring the occurrence of GvHD. Nagasawa et al. [17]. discovered that T cell-specific $CD3^+$ $CD8^+$ EVs and $CD3^+$ $HLA\text{-}DR^+$ EVs levels were strongly correlated with the risk of aGvHD. By examining the EVs derived from the peripheral blood of 20 patients who underwent HSCT, they ascertained that the levels of $CD3^+$ $CD4^+$ EVs and $CD3^+$ $CD8^+$ EVs were correlated explicitly with the counts of $CD4^+$ and $CD8^+$ T lymphocytes, respectively. Meanwhile, $CD3^+$ $CD8^+$ EVs and $CD3^+$ $HLA\text{-}DR^+$ EVs escalated in patients with GvHD and were more specifically competent in reproducing the persistence of GvHD than soluble interleukin 2 receptor (sIL-2R). Engraftment syndrome (ES) is a non-infectious fever syndrome that typically occurs during the recovery of neutrophils after HSCT, and its clinical manifestations include fever, rash, and pulmonary infiltration (68–69). This syndrome can occur in patients undergoing both autologous and allo-HSCT. Because the symptoms of ES are similar to those

of aGvHD, it was once called super-acute GvHD [17]. In engraftment syndrome, sIL-2R was significantly elevated, whereas $CD3^+HLA\text{-}DR^+$EVs were not. Furthermore, ferritin and sIL-2R were conspicuously elevated in pre-transplant hemophagocytic syndrome; However, $CD3^+$ $HLA\text{-}DR^+$ EVs showed little change. $CD3^+$ $CD4^{+-}$, $CD3^+$ $CD8^{+-}$, and $CD3^+$ $HLA\text{-}DR^{+-}$ EVs can effectively stimulate cell-mediated immune responses. The proportion and functional alterations of T cell subsets exert significant roles in the occurrence and development of GvHD. Thus, detecting the variations of EV subsets derived from T cells might constitute a more direct and practical approach for monitoring the occurrence of GvHD. In contrast to other conventional biomarkers, $CD3^+$ $CD8^+$ and $CD3^+$ $HLA\text{-}DR^+$ may exhibit superior performance in in monitoring and assessing aGvHD. The researchers independently developed a novel EV technology platform for automatic acoustic capture and separation to overcome the technical bottleneck of separating EVs from small plasma volumes. They found that the amount of EV captured in plasma increased more than two-fold in most patients after allo-HSCT [18]. The expression of platelet-specific miR-142-3p in EVs after transplantation is closely correlated with the expected platelet count dynamics. More importantly, the authors also found that some miRNAs in plasma EVs were strongly correlated with the occurrence and development of GvHD. These research results imply that the monitoring of circulating EVs is expected to become a valuable biomarker for the occurrence and development of GvHD and the prognosis of treatment (Fig. 3).

The membrane antigen components of circulating EVs and the diagnostic application of GvHD

Studies have demonstrated that specific membrane components of EVs, such as lipids and membrane proteins, as well as their contents, including nucleotides and metabolites, are closely associated with the occurrence and progression of diseases. Recently, studies have shown that surface antigens of EVs have a potential correlation with the occurrence of aGvHD (70–71). Baur et al. [72]. discovered that circulating EVs in the plasma of patients after allo-HSCT, expressed PD-L1 and $PD\text{-}L1^+$ EVs, attained the peak of PD-L1 expression 6 weeks after allo-HSCT. The expression of PD-L1 in these EVs was associated with their capacity to inhibit T cells and the emergence of GvHD. The development of aGvHD is closely related to the appearance of EVs with high PD-L1 expression in allo-HSCT post-transplantation. Moreover, the expression level of PD-L1 in patients is positively correlated with the severity of GvHD. And PD-L1 expression levels will significantly decrease only after successful treatment of GvHD. The above results suggest that PDL1 EVs may be used to monitor the clinical efficacy of GvHD treatment. In another study, Lia et al. [70]. detected serum EVs of 41 patients with multiple myeloma who received allo-HSCT using 13 antibodies against specific membrane proteins and found that the membrane antigens CD146, CD31, and $CD140\text{-}\alpha^+$ EVs had a strong correlation with the appearance of aGvHD. Among them, CD146 (MCAM-1) increased the risk of aGvHD by nearly 60%, whereas CD31 (PECAM-1) and CD140 (PDGFR) decreased the risk of almost 40% and 60%, respectively. The emergence of EVs with differential expression of membrane antigens indicates that the risk of aGvHD significantly increases for patients after transplantation. The variations of EV subpopulations may precede the clinical manifestations of GvHD, offering crucial early warning information for the early prevention and clinical intervention of GvHD. In a follow-up study, Lia et al. [71]. further observed and monitored the expression profiles of CD146, CD31, CD140a, CD120a, CD26, CD144, CD30, and their miRNA loading on EVs in 32 consecutive patients, which showed a significant correlation with the pathogenesis of aGvHD. They also found that the expression profile of EVs and plasma biomarkers (such as ST2, sTNFR1, and REG3a) potentially correlated with aGvHD. The combination of CD146, sTNFR1, and miR-100 or miR-194 in EVs was closely related to the pathogenesis of aGvHD. Its diagnostic efficacy, indicated by an AUROC (area under the receiver operating characteristic curve) > 0.975, suggested that the expression profile of EVs was expected to be a promising biomarker for the early diagnosis of aGvHD. The above studies have demonstrated that the abnormal alterations in the expression profiles of membrane antigen components of EVs and their carried contents, such as nucleotides or proteins, are closely associated with the disease occurrence and progression of GvHD. The individual or combined detection of EV components is anticipated to become an excellent biomarker for the early diagnosis or prognosis monitoring of aGvHD.

The nucleotide components of circulating EVs and their diagnostic application in GvHD

Recent studies have shown that miRNAs encapsulated by circulating EVs in patients are expected to be promising biomarkers for the early monitoring of aGvHD and cGvHD. Lim et al. [18]. showed that miRNAs in plasma EVs can predict GvHD and correlate with disease severity and patient survival. The authors utilized acoustic fluidic capture technology to capture EVs in plasma samples from 20 consecutive patients with high-risk/relapsed hematological malignancies 12 weeks pre- and post-allo-HSCT. They ascertained that the number of EVs in most patients increased by more than 2-fold after transplantation. Through the analysis of the miRNA expression profiling of EVs, the expression of miR-15b-3p, miR-30a-5p,

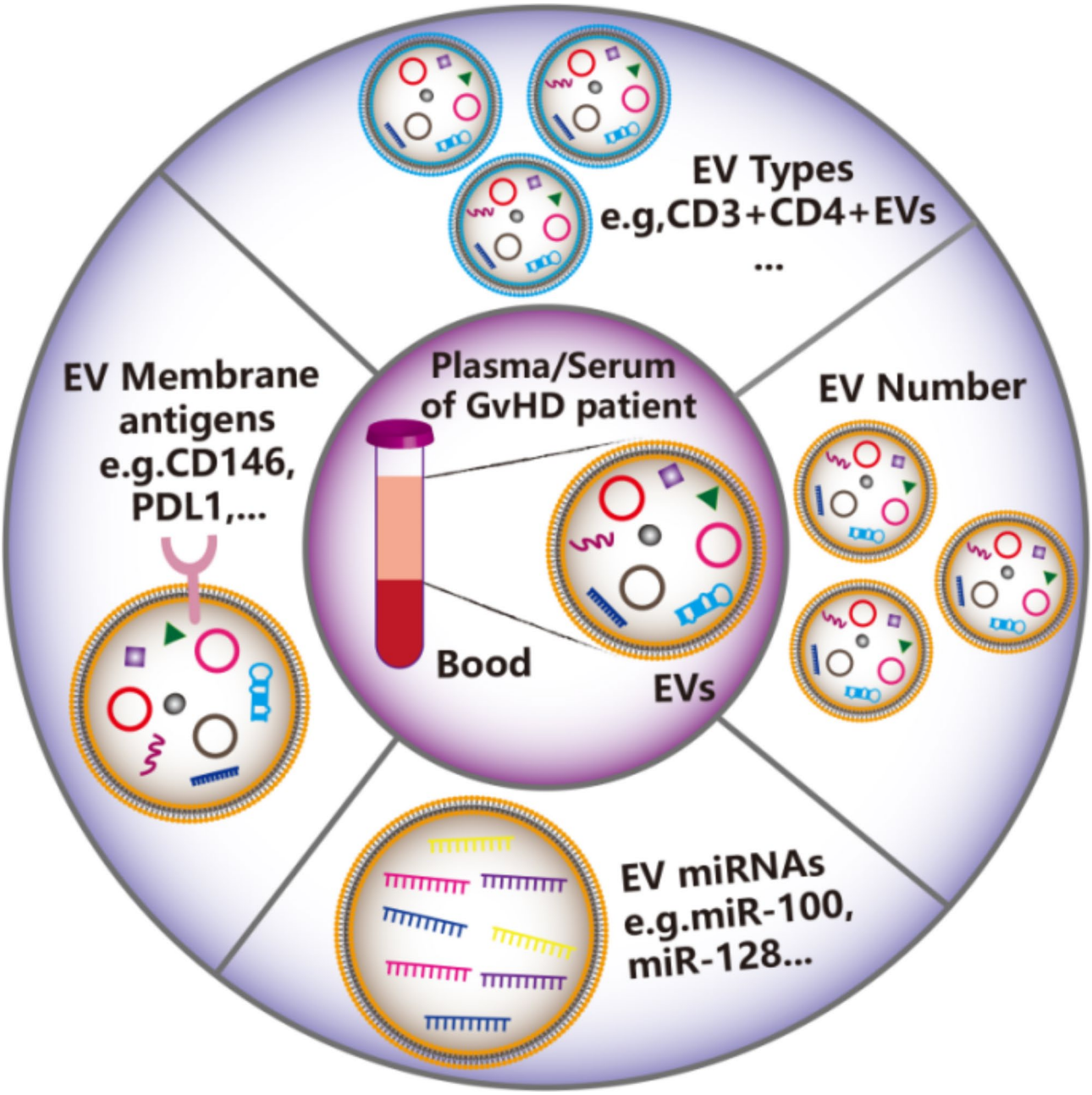

Fig. 3 Diagnostic application of EVs in GvHD

Current studies demonstrate that EVs in the plasma or serum of GvHD patients are expected to become significant markers for diagnosing acute and chronic GvHD. By detecting changes in the quantity, subtype, and content of EVs in patients' blood, researchers can effectively predict the onset and progression of GvHD. This approach provides an important reference basis for clinical practice. GvHD, Graft-versus-host hisease; EVs, Extracellular vesicles; CD146, Cluster of Differentiation 146; PDL-1, Programmed cell death-ligand 1; miR-100, miRNA-100; miR-128, miRNA-128

miR-130a-3p, miR-145-5p, and miR-342-3p in plasma EVs was conspicuously augmented after transplantation; in contrast, the expression of miR-18a-5p, miR-92b-3p, miR-93-5p, miR-141-3p, and miR-486-5p was significantly diminished. Importantly, they found that upregulated miRNAs in plasma EVs were positively correlated with infection and GvHD, respectively, while the downregulated miRNAs were negatively correlated with these complications. Furthermore, platelet-specific miR-142-3p expression was also associated with expected platelet count dynamics in graft EVs. This study reveals that it is crucial to construct a rapid separation technology platform for plasma EVs based on acoustic principles and elucidate EV miRNA's important significance as a potential biomarker for early prevention and diagnosis of infection or GvHD after allo-HSCT. In another

study, Lacina et al. [73]. performed the detection of miRNA expression profiles in 3 patients with cGvHD and 4 patients without cGvHD and no disease symptoms and identified that there were three differentially expressed miRNAs in patients with cGvHD, namely hsa-miR-29c-3p, hsa-miR-374b-5p, and hsa-miR-630. Among them, the latter two levels were significantly decreased by 2.7-fold and 4.1-fold, respectively, while the expression of hsa-miR-29c-3p was elevated considerably by 5.8-fold. These results suggest that the miRNA profiles of plasma EVs might be used as markers for the pathogenesis of cGvHD.

In 2005, the new consensus standard classification established by the National Institutes of Health (NIH) defined aGvHD that occurs more than 100 days after transplantation as late-onset aGvHD (LA GvHD) [39]. According to this NIH definition, approximately 20–40% of patients with cGvHD may be reconsidered as LA GvHD. LA GvHD is also associated with higher non-relapsed mortality after allo-HSCT compared to non-GvHD [74]. The underlying mechanism of LA GvHD remains poorly understood, and the identification of differentially expressed miRNAs in EVs is of significant importance in revealing the role of miRNAs in this disease. Yoshizawa et al. [75]. collected EVs from the plasma of 5 patients with LA GvHD, 5 patients with non-GvHD, and 8 healthy controls and performed miRNA expression profiling. They identified 55 miRNAs with differential expression between LA GvHD and non-GvHD. Among them, through conducting further verification on the 10 miRNAs (such as miR-19a, miR-30c, miR-125b, miR-128, miR-142-3p, miR-191, miR-193a, miR-193b, miR-423-5p, and miR-574-3p) that were most differentially expressed, miR-128 was upregulated to a significantly greater extent in the pathogenesis of LA GvHD, with an area under the curve (AUC) of 0.975. Previous studies have indicated that miR-128 shows abnormal expression in the tissues and blood samples of some patients with malignant tumors and is associated with tumor progression. Moreover, MirTarBase analysis showed that miR-128 target genes were involved in the immune system and inflammatory response. These results further imply that miR-128 present within EVs is expected to act as a novel and non-invasive biomarker for the early diagnosis of LA GvHD (Table 1). Overall, the attribute of circulating EV miRNA constitutes a non-invasive and highly promising biomarker for the early diagnosis of acute, chronic, and late-onset GvHD.

Circulating EVs and the medication and prognosis monitoring of GvHD

Treatment with rabbit anti-T lymphocyte globulin (ATLG) is an effective strategy for the prevention of GvHD, and its effectiveness in preventing GvHD has

Table 1 Diagnostic value of EVs in GvHD

EV Types	Cargoes	Change	Clinical outcome	Ref
Plasma EVs	miR-15b-3p, miR-30a-5p, miR-130a-3p, miR-145-5p, and miR-342-3p miR-18a-5p, miR-92b-3p, miR-93-5p, miR-141-3p, and miR-486-5p	↑ ↓	The miRNAs in plasma EVs can predict GvHD and are closely associated with the severity of the disease and the survival of the patient	[18]
Plasma EVs	PEVs and EryEVs	↑	PEVs and EryEVs for assess the endothelial injury and procoagulant activity of allo-HSCT survivors	[67]
Plasma EVs		↑	The counts of TEVs and EryEVs are closely related to aGvHD	[16]
Serum EVs	$CD3^+CD4^+$-, $CD3^+CD8^+$-, $CD3^+HLA\text{-}DR^+$EVs	↑	The levels of T-cell-specific $CD3^+CD8^+$- and $CD3^+HLA\text{-}DR^+$ EVs are strongly correlated with aGvHD	[17]
Plasma EVs	The membrane antigens CD31, CD140-α, and CD146 of EVs	↑	$CD31^+$-, CD140-α^+-, and $CD146^+$- EVs are closely related to aGvHD	[70]
Plasma EVs	CD146, sTNFR1, and miR-100 or miR-194	↑	CD146, sTNFR1, and miR-100 or miR-194 are closely related to aGvHD	[71]
Plasma EVs	$PD\text{-}L1^+$EVs	↑	$PD\text{-}L1^+$ EVs are strongly correlated with aGvHD	[72]
Serum EVs	$CD69^+$ EVs	↑	$CD69^+$ EVs are associated with aGvHD risk	[76]
Plasma EVs	miR-29c-3p, miR-630, and miR-374b-5p	↑ ↓	The miRNA spectrum in plasma EVs for the diagnosis of cGvHD	[73]
Plasma EVs	miR-128	↑	Employed for the early non-invasive diagnosis of LA GvHD	[75]

Abbreviations: EVs, Extracellular vesicles; ROC, Receiver operator characteristic curve; Ref, Reference; GvHD, Graft-versus-host disease; aGvHD, Acute GvHD; cGvHD, Chronic GvHD; LA GvHD, Late-onset aGvHD; miR, miRNA; EryEVs, Erythrocyte-derived EVs; PEVs, Platelet-derived EVs; PD-L1, Programmed cell death-ligand 1; sTNFR1, Soluble tumor necrosis factor receptor 1

been demonstrated in randomized studies. However, its impact on relapse remains a significant concern for broader application. Therefore, the early adjustment of the ATLG dosage in line with the host characteristics is expected to minimize its side effects (such as immune reconstitution, recurrence, and infection), representing an effective means to improve the clinical intervention of ATLG. Storci et al. [76]. found that the serum ATLG activity level was significantly decreased in 23 patients with GvHD. Meanwhile, the concentration of $CD69^+$ EVs in patients with GvHD increased at the time before infusion. The researchers obtained consistent results in 12 other validation cohorts of HSCT patients who received ATLG treatment. These results suggest that high levels of serum $CD69^+$ EVs are significantly correlated with an augmented risk of GvHD, and are anticipated to be an effective means for customizing ATLG dose monitoring for individualized prevention of GvHD.

Therapeutic effects of EVs in GvHD and its related complications

Long-term suppression of the host immune system and methods to improve transplant tolerance remain the main challenges in organ transplantation and GvHD [77]. MSC-EVs have been used in the treatment of a variety of immune diseases. Allogeneic human MSC products can be used in the clinical treatment of GvHD, but clinical application data indicate that these MSC products have certain limitations in treatment practice. Many patients with aGvHD respond to cell therapy of human bone marrow/umbilical cord MSCs/stem cells. However, the mechanism of these cells to improve complications related to aGvHD remains to be clarified. MSC-EVs are due to their unique biological distribution and significant dose-dependent therapeutic effects. The unique immunomodulatory properties of MSC-EVs make them particularly important in grafts in which the immune system is overactive, such as allo-HSCT. Recent studies have revealed that MSC-EVs have promising therapeutic effects in preclinical and clinical studies. Some specific miRNAs, membrane proteins, membrane lipids, and soluble factor binding components carried by MSC-EVs can treat GvHD and its related complications through various mechanisms, such as regulation of T cell-mediated adaptive immunity and APC-mediated innate immunity and direct regeneration of injured target organs.

Therapeutic effects of EVs in aGvHD

MSCs have been utilized in treating aGvHD, cGvHD, and their associated complications, as they discharge a wide range of mediators, including immunosuppressive molecules, growth factors, chemokines, and EVs (Tables 2 and 3) [89]. Studies have investigated the effects of MSC administration on aGvHD, with differing results. Increasing evidence suggests that MSC-derived cytokines, EVs, and other components are critical in alleviating GvHD.

Table 2 Application of MSC-EVs in treating GvHD

Source of EVs	Cargoes	Target genes or pathways	Effects	Ref
hBMMSC-EVs	-	-	-	[25]
hucMSC-EVs	ATOs	mTOR-autophagy pathway	Promote the polarization of macrophage M1 to M2 type and reduce the severity of aGvHD	[29]
hucMSC-EVs, mBMMSC-EVs	miR-204	IL-6/IL-6R/STAT3 pathway	Amelioration of cGvHD-associated dry eye via regulation of M2 macrophage polarization	[78]
hBMMSC-EVs	miR-125a-3p CXCL12	-	Inhibit the functional differentiation of naive T cells into effector T cells and maintain $CD4^+$ $CD25^+$ $Foxp3^+$ Treg in aGvHD	[79]
hBMMSC-EVs	-	-	Inhibited $CD4^+$ T cells and Th17 cells, and induced Tregs in cGvHD	[80]
mBMMSC-EVs	miR-21	Targeted the PTEN/AKT/Foxp3 pathway	Improved immunomodulatory properties of regulatory Tregs in cGvHD	[81–82]
hBMMSC-EVs	CD73	-	Promoted adenosine-based immune suppression in x-GvHD	[83]
hPMSC-EVs	-	-	Promoted the treatment of chronic skin GvHD	[84]
ihESC-EVs	-	-	Enhanced Tregs production via an APC-mediated pathway in x-GvHD	[85]
hucMSC-EVs mb-MSC-EVs	miR-223	ICAM-1	Reduced the migration of donor T cells and the expression of inflammatory cytokines in aGvHD	[86]
hucMSC-EVs	-	-	Regulating immune response in aGvHD	[87]
hBMMSC-EVs	-	-	Regulate the subsets of DCs and T cells as well as their functions, and restrain the inflammatory response in aGvHD	[88]

Abbreviations: EVs, Extracellular vesicles; apoEVs, EVs released by apoptotic cells; Ref, Reference; miR, miRNA; MSC/MSCs, Mesenchymal Stromal/Stem Cells; mBMMSCs, Murin bone marrow MSCs; hBMMSCs, Human bone marrow MSCs; HucMSCs, Human umbilical cord MSCs; hPMSCs, Human placenta MSCs; ihESCs, Immortalized human embryonic stem cell-derived MSCs; DCs, Dendritic cells; ATOs, Arsenic trioxides; GvHD, Graft-versus-host disease; aGvHD, Acute GvHD; cGvHD, Chronic GvHD; x-GvHD, Human-into-mouse xenogeneic GvHD; Tregs, Regulatory T cells; ICAM-1, Intercellular adhesion molecule-1

Table 3 Ongoing clinical trials concerning MSCs and MSC-EVs for the intervention of GvHD and its associated complications

No.	Study title*	Status	Condition	Intervention	NTC number
1	Sequential administration of WJ-MSCs for the treatment of GvHD refractory to second-line treatment	Not yet recruiting; Phase 1/2	aGvHD	I.V. of WJ-MSCs	NCT06304025
2	Umbilical cord mesenchymal stem cells as first-line treatment for patients with acute graft versus host disease	Recruiting; Not applicable	aGvHD	Infusion of hUCMSCs	NCT05531266
3	Mesenchymal stem cells for the treatment of refractory acute graft versus host disease	Unknown status; Phase 2	aGvHD	Infusion of hBMMSCs	NCT01765634
4	Interferon γ-primed mesenchymal stromal cells as prophylaxis for acute graft v host disease	Suspend; Phase 1	aGvHD	Infusion of hBMMSCs	NCT04328714
5	Placenta-derived mesenchymal stem cells and placenta-derived mesenchymal stem cells exosomes in the treatment of acute GvHD	Not yet recruiting; Phase 1/2	aGvHD	Infusion of hPMSC-sEVs	IRCT20140818018842N40
6	Treatment of severe acute GVHD after allogeneic hematopoietic stem cell transplantation with steroids versus MSC and steroids. A prospective double-blind placebo-controlled randomized phase III trial	Recruiting; Phase 3	aGvHD	Infusion of MSCs	NL-OMON41587
7	MSC for severe aGVHD	Unknown status; Phase 2/3	Severe aGVHD	hMSCs	NCT03631589
8	Safety and efficacy of UC-MSC in patients with acute severe graft versus host disease	Unknown status; Phase 1/2	Acute severe GvHD	Infusion of hUCMSCs	NCT01754454
9	Infusion of allogeneic bone marrow mesenchymal stem cells in patients with steroid-refractory GVHD	Completed; Phase 1/2	SRaGvHD	Infusion of hBMMSCs	NCT02824653
10	Treatment of steroid-resistant GVHD by infusion MSC	Completed; Phase 1/2	SRaGvHD	Infusion of hMSCs	NCT00827398
11	Efficacy and safety of UC-MSCs for the treatment of steroid-resistant aGVHD following allo-HSCT	Completed; Phase 3	SRaGvHD	Infusion of hUCMSCs	NCT04738981
12	Mesenchymal stem cells as first treatment line for resistant acute graft versus host disease	Unknown status; Phase 2	SRaGvHD	Infusion of hBMMSCs	NCT02770430
13	Extracorporeal photopheresis and Mesenchymal stem cell infusion for GVHD	Not yet recruiting; Phase 2	HR/SRa GvHD	I.V. of allogeneic MSCs	NCT05333029
14	Dose-escalation trial of mesenchymal stromal cells in patients with medical xerostomia	Not yet recruiting; Phase 1	Xerostomia (GvHD)	Local injection of BMMSCs	NCT06392711
15	BMT autologous MSCs for GvHD	Completed; Phase 1	a/cGvHD	I.V. of autologous BMMSCs	NCT02359929
16	MSCs combined with CD25 monoclonal antibody and calcineurin inhibitors for treatment of steroid-resistant aGVHD	Unknown status; Phase 2/3	aGvHD	Infusion of BMMSCs	NCT02241018
17	A Study of CYP-001 in combination with corticosteroids in adults with high-risk aGvHD	Recruiting; Phase 2	SRaGvHD	Infusion of CiPSC-MSCs	NCT05643638
18	Addition of cord blood tissue-derived mesenchymal stromal cells to ruxolitinib for the treatment of steroid-refractory cells acute graft versus host disease	Recruiting; Early Phase 1	SRaGvHD	Infusion of hUCMSCs	NCT04744116
19	Mesenchymal stromal cells (MSCs) for the treatment of graft versus host disease (GVHD)	Unknown status; Phase 1	Severe (Grade II-IV) SRaGvHD	Infusion of hMSCs	NCT01764100
20	A prospective study of remestemcel-L, ex-vivo cultured adult human mesenchymal stromal cells, for the treatment of pediatric participants who have failed to respond to steroid treatment for acute graft-versus-host disease (aGVHD)	Completed; Phase 3	SRaGvHD	Infusion of hMSCs	NCT02336230
21	Mesenchymal stem cells (MSCs) for steroid refractory acute GVHD (SR-aGVHD)	Withdraw; Phase 1	SRaGvHD	Infusion of hBMMSCs	NCT05443464
22	Safety and efficacy study of adult human mesenchymal stem cells to treat acute graft versus host disease (GVHD)	Completed; Phase 2	GvHD	Infusion of hMSCs	NCT00136903

Table 3 (continued)

No.	Study title*	Status	Condition	Intervention	NTC number
23	Clinical research of umbilical cord–derived mesenchymal stromal cells in the prophylaxis of graft-versus-host disease after HLA-haploidentical stem-cell transplantation	Recruiting; Phase 4	GvHD	Infusion of hUCMSCs	ChiCTR1900022292
24	Efficacy and safety study of allogenic mesenchymal stem cells for patients with chronic graft versus host disease	Unknown status; Phase 2/3	cGvHD	Bone marrow injection of hMSCs	NCT01526850
25	MSC for the treatment of cGVHD after allo-HSCT	Unknown status; Phase 2	cGvHD	Infusion of BMMSCs	NCT04692376
26	Effect of PMSCs and their secretome for the treatment of GvHD	Completed; Phase 3	GvHD	Infusion /skin spray of hPMSCs and their secretome	NCT06469411
27	Mesenchymal stem cells for the treatment of refractory chronic graft versus host disease	Unknown status; Phase 2	cGvHD	Infusion of BMMSCs	NCT01765660
28	Treatment of chronic oral graft versus host disease with human umbilical cord mesenchymal stem cell dressing	Recruiting; Phase 4	Oral cGvHD	hUCMSCs dressing	NCT06149832
29	Effect of UMSCs derived exosomes on dry eye in patients with cGVHD	Completed; Phase 1/2	cGvHD-related DES	Eye drops of hUCMSC-sEVs	NCT04213248

* Study titles are indicated as listed on ClinicalTrials. https://clinicaltrials.gov/. and https://trialsearch.who.int/ Accessed 30 August 2024

Abbreviations: EVs, Extracellular vesicles; sEVs, Small EVs; GvHD, Graft-versus-host disease; aGvHD, Acute GvHD; cGvHD, Chronic GvHD; LA GvHD, Late-onset aGvHD; HRaGvHD, High-Risk aGvHD; SRaGvHD, Steroid-refractory aGvHD; x-GvHD, Human-into-mouse xenogeneic GvHD; MSC/MSCs, Mesenchymal Stromal/Stem Cells; WJ-MSCs, Wharton's jelly MSCs; BMMSCs, Bone marrow MSCs; hMSCs, Human MSCs; hUCMSC/UC-MSCs/UMSCs, Human umbilical cord MSCs; CiPSC-MSCs, Cymerus induced pluripotent stem cell (iPSC)-derived MSCs; hPMSCs, Human placenta MSCs; ihESCs, Immortalized human embryonic stem cell-derived MSCs; mb-MSCs, Murine compact bone MSCs; MDSCs, Myeloid-derivative-suppressor cells; BMT, Bone marrow transplantation; IFN-γ, Interferon gamma; HLA, Human leukocyte antigen; DES, Dry eye syndrome

Kordelas et al. [25]. were the first to report a clinical case of mucosal GvHD after PBSCT for myelodysplastic syndrome. A female patient developed moderate mucosal GvHD after her first allo-HSCT using PBSC from an HLA-identical female donor for myelodysplastic syndrome at age 13. At age 20, she was diagnosed with secondary AML and underwent a second allogeneic stem cell transplant with PBSC from a male donor of the same HLA, resulting in hyperacute skin GvHD (Grade 4). The patient experienced severe intestinal and cutaneous GvHD, treated with steroids and MSC-EVs. Shortly after starting MSC-EV therapy, the pro-inflammatory cytokine response in peripheral blood mononuclear cells significantly decreased, leading to a marked improvement in GvHD symptoms: diarrhea severity was notably reduced post-treatment. Skin and mucosal GvHD also showed significant improvement within 2 weeks and remained stable even after 4 months of MSC-EV treatment. Consequently, steroid dosage was reduced from 125 mg to 30 mg per day. The patient's condition stayed stable for several months. Although the patient ultimately passed away from pneumonia seven months after receiving MSC-EVs, these promising results suggest that MSC-EVs may offer a novel and safe approach for treating refractory GvHD and other inflammation-related disorders.

EVs improve GvHD by regulating the function of $CD4^+$ and $CD8^+$ T cells

Cord blood-derived multipotent stem cells (CB-SCs) were found to improve the development of the GvHD response in xenohuman PBMC-induced NSG mouse models in vivo. Mechanistically, CB-SCs improve allogeneic response in vitro, an effect associated with CB-SC-derived EVs that suppress the proliferation and activation of T cells in allogeneic mixed lymphocyte cultures [90]. The dysfunction of $CD4^+$ T cells in GvHD may be an essential factor aggravating GvHD. A recent study by Li et al. [91]. revealed that hucMSC-EVs can efficaciously reduce damage from GvHD by alleviating disorders of redox metabolism and inflammatory cytokine bursts in $CD4^+$ T cells. Moreover, the authors discovered that miR-16-5p, which is abundantly present in hucMSC-EVs, can alleviate endoplasmic reticulum stress and decrease the apoptosis of $CD4^+$ T cells through targeted inhibition of the ATF6/CHOP signaling axis, facilitate the development of $CD4^+$ $IL\text{-}10^+$ T cells, and restore immune homeostasis during the pathogenesis of GvHD. Wang et al. [87]. found that hucMSC-EV treatment could regulate the immune response to prevent life-threatening aGvHD. Further investigations disclosed that the infusion of EVs conspicuously mitigated the clinical score and pathological impairment in mice afflicted with aGvHD and diminished their mortality rate. After EV treatment, the frequency and absolute quantity of $CD3^+CD8^+$ T cells

were significantly decreased, whereas the proportion of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells escalated; the concentrations of IL-2, IFN-γ, and TNF-α in serum dropped, while the level of IL-10 in serum ascended. In vitro experiments showed that EVs could dose-dependently inhibit mitogen-induced splenocyte proliferation, inhibit the expression of pro-inflammatory cytokines levels of IL-2, IFN-γ, and TNF-α, and promote the expression of IL-10. The results of this study also suggested that hucMSC-EVs are an ideal alternative for the prevention of aGvHD after allo-HSCT.

The miRNAs within EVs derived from bone marrow MSCs play a crucial role in regulating immune responses; however, the function of miRNAs in treating aGvHD remains undefined. Liu et al. [86] uncovered through high-throughput sequencing that miR-223 was strikingly highly expressed in both hucMSC-EVs and mBMSC-EVs and disclosed that miR-223 derived from MSC-EVs could diminish donor T cell migration by targeting ICAM-1, thereby mitigating the symptoms of aGvHD. Mechanistic studies showed that miR-223 could inhibit ICAM-1 expression in mouse lymphatic endothelial cells to inhibit T-cell adhesion and migration, and miR-223 AgomiR infusion could alleviate the clinical symptoms of aGvHD, reduce donor T cell infiltration into the spleen, liver, and intestinal tract, and reduce the expression of the inflammatory cytokines IFN-γ, TNF-α, and IL-17. Fujii et al. [79]. systematically administered human bone marrow MSC-EVs not only prolonged the survival of aGvHD mice but also reduced the damage of various target organs of GvHD. In GvHD mice treated with EVs, $CD4^+$ and $CD8^+$ T cells were inhibited, and the proportion of $CD62L^-$ $CD44^+$ and $CD62L^+$ $CD44^-$T cells was reduced, suggesting that EVs can inhibit the functional differentiation of T cells from the naïve phenotype to the expression of the effector phenotype. In addition, EVs also preserved the $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T-cell population. Amarnath et al. [83]. found that CD73-expressing BMMSC-EVs mediated adenosine-based immunosuppression and could improve x-GvHD. The authors first established an x-GvHD model of human $CD4^+$ Th1 cell death and explored immunomodulation using clinical-grade BMMSC products. BMMSCs could reverse the established, lethal x-GvHD by significantly inhibiting the Th1 cell effector function. The implantation of BMMSCs was limited to the lungs without affecting regulatory T cells, suggesting that the paracrine mechanism of BMMSCs was the key mechanism mediating this effect. Further studies found that serum CD73-expressing MSC-EVs in BMMSCs recipients increased significantly and promoted adenosine accumulation in vitro. BMMSC-mediated immunomodulatory effects were wholly abolished by drug therapy with adenosine A2A receptor antagonists. To investigate the underlying mechanisms and reveal their potential clinical relevance, we examined the content of MSC-EVs in serum samples collected from patients before and after treatment with BMMSCs. We found that MSC-EVs expressing CD73 promoted adenosine accumulation in serum samples from patients after treatment with BMMSCs. BMMSCs effectively modulate experimental GvHD by promoting adenosine-based immunosuppression through paracrine activation.

EVs improve GvHD by regulating the function of regulatory T cells

Zhang et al. [85]. found that EVs derived from immortalized human embryonic stem cell-derived MSCs increased T-reg production in mice with allogeneic skin grafts but not in engrafted mice. MSC-EVs were jointly utilized in combination with mouse splenic $CD4^+$ T cells activated by anti-CD3/CD28 monoclonal antibodies or splenic $CD11c^+$ cells that are rich in allogeneic antigen-presenting cells (APCs) to determine whether mouse $CD4^+$ $CD25^+$ T cells or $CD4^+$ $CD25^+$ $Foxp3^+$ cells could be induced. MSC-EVs have also been utilized in a lethal human-SCID chimeric mouse GvHD model. In this model, human PBMC was infused into irradiated NSG mice to induce GvHD. MSC-EVs can induce $CD4^+$ $CD25^+$ T cells or $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs activated by allogeneic $CD11c^+$ cells rather than those activated by anti-CD3/CD28 monoclonal antibodies. This induction effect demonstrates a dose-dependent relationship with EVs. MSC-EVs attenuated symptoms of GvHD and increased survival in a murine model of a GvHD induced by a human antimouse $CD4^+$ T cell effector stimulated by transplanted human APC. The levels of human $CD4^+$ $CD25^+$ $CD127^{low/-}$ Tregs in surviving MSC-EVs-treated mice were significantly higher than those in surviving mice treated with the TNF inhibitor etanercept. MSC-EVs facilitate the generation of regulatory T cells in vivo and in vitro via the APC-mediated pathway.

EVs improve GvHD by regulating the function of other immune cells and the inflammatory response

MSC-EVs have been found to alleviate GvHD and preserve the graft-versus-leukemia (GVL) effect by inhibiting the immunomodulatory functions of DCs, macrophages, and T lymphocytes in allo-HSCT animal models [92]. Treatment with MSC-EVs can effectively reduce the clinical symptoms of GvHD patients after HSCT and significantly improve the survival rate of HSCT recipients. Studies in GvHD patients have shown that MSC-EVs inhibit the release of IFN-γ and TNF-α through activated NK cells, thus reducing the lethal function and inflammatory response of NK cells [93]. MiRNA microarray screening showed that miR-125a-3p was the most significantly upregulated in EV, and its downstream target genes were closely related to the inhibition

of T-cell proliferation. Recently, Li et al. [88]. found that hBMMSC-EVs could reduce pathological damage caused by aGvHD and promote the survival of mice by regulating the function of DCs and T cell subsets and inhibiting the inflammatory response of mice. The intervention of EV resulted in a decrease in the expression of IL-2, IFN-γ, and TNF-α, an increase in the expression of IL-10, reduced body weight loss, clinical scores, and mortality in aGvHD mice. Further studies found that EV treatment resulted in a 7- to 8-fold increase in $CD8\alpha^+$ and $CD11b^+$ conventional DCs and changed the subsets of helper T cells and Tregs, reducing the proliferation of cytotoxic T cells, which was conducive to inflammation suppression in aGvHD mice. Because human experimentation is strictly restricted by moral, ethical, legal, and other factors, the human-mouse GvHD model (xenogeneic GvHD, x-GvHD) has become an ideal experimental tool for GvHD research because of its reliability and stability.

Therapeutic effects of EVs in cGvHD and GvHD-related complications

cGvHD is a systemic, multi-organ syndrome; the main affected target organs include the skin, eyes, liver, and gastrointestinal tract. Norooznezhad et al. [84]. reported a second case of EV treatment in a patient with cutaneous cGVHD, showing clinically acceptable results for both the team and the patient. The results remained stable for 4 months without recurrence. This study only investigated the treatment of hPMSCs with EVs. The authors suggest that studies on BMMSC from other sources, such as bone marrow, should be carried out to treat cGvHD. In addition, changes in the environment of BMMSCs could also be considered another variable in future studies. The incidence and severity of skin ulcers, wounds, keratinization, and atrophic lesions were reduced, and wound healing was observed. Additionally, there was a significant improvement in the degree of sclerosis and dryness of the skin after treatment. Dry eye syndrome (DES) associated with GvHD is characterized by extensive inflammatory destruction of the ocular surface, leading to unbearable pain and visual impairment. Current clinical treatments have limited efficacy. Zhou et al. [78]. reported that MSC-EVs can relieve GvHD-related DES by inhibiting inflammation and improving epithelial cell recovery in mice and humans. In a prospective clinical trial, the authors found that hucMSC-EVs significantly improved the symptoms of 28 patients with refractory GvHD-related DES. After intervention with hucMSC-EVs, the fluorescein score was reduced, the tear film break-up time was prolonged, tear secretion was increased, and the OSDI score was reduced. Similarly, in a mouse model of GvHD-related DES-induced by benzalkonium chloride (BAC), the symptoms of DES were effectively alleviated after treatment with mouse bone marrow MSC-EVs (mBMMSC-EVs). Mechanistic studies found that a miR-204 molecule enriched in MSC-EVs can target the IL-6/IL-6R/STAT3 signaling axis to reprogram pro-inflammatory M1 macrophages into immunosuppressive M2 macrophages to attenuate DES. To evaluate the safety and efficacy of MSC-EVs in a transplant patient population, Lightner et al. [94]. used MSC-EVs to treat isolated intestinal transplant patients ($n = 2$), liver allograft patients ($n = 2$), modified multi-visceral transplantation patients ($n = 3$), and GvHD patients ($n = 2$). The results showed that the clinical symptoms of all patients improved within 24 h after EVs treatment, the serological laboratory evaluation indicators, the lung symptoms and skin manifestations of GvHD improved, and the graft inflammation/rejection was entirely resolved histologically within 7 days after administration.

$CD4^+$ T cells and Th17 cells play a crucial role in the pathogenesis of cGvHD [47, 49]. Lai et al. [80]. found that hBMMSC-EVs could effectively prolong the survival of cGvHD mice, reduce the clinical and pathological scores of mice, and inhibit the skin, lung, and liver fibrosis process by exerting powerful immunomodulatory effects on GvHD effector T cells. In the process of EVs intervention in cGvHD, $CD4^+$ T cell activation and lung infiltration were reduced, the expression of Th17 cell-related transcription factors and pro-inflammatory cytokines was significantly reduced, and the pathogenic T cells expressing IL-17 and regulatory cells inducing IL-10 expression were inhibited. EVs blocked Th17 cell differentiation of Th17 cells from PBMCs of healthy donors and patients with cGvHD in vitro and improved the Treg phenotype. hBMMSC-EVs could enhance the survival of patients with cGvHD and their pathological injury by inhibiting Th17 cell proliferation and activity of Th17 cells and inducing Treg expression. Early administration of MSC-EVs to inhibit persistent Th17 cell-mediated inflammation may block the progression of the fibrotic stage by exacerbating overactive inflammation levels. Therefore, MSC-EVs are expected to be effective reagents for the prevention or treatment of cGvHD.

Engineering EVs for the treatment of GvHD and its related complications

Owing to its remarkable biocompatibility and physical and chemical stability, EVs are regarded as outstanding means of transportation by many researchers. Both hucMSC-EVs and arsenic trioxides (ATOs) have been shown to treat aGvHD through immunomodulation. In addition to immunomodulation, hucMSC-EVs have a unique drug delivery function, which is believed to improve their activity (Fig. 4). ATOs are effective drugs for the treatment of acute promyelocytic leukemia. In addition to the direct intervention of ATOs in acute promyelocytic leukemia, recent studies have also found that it can play

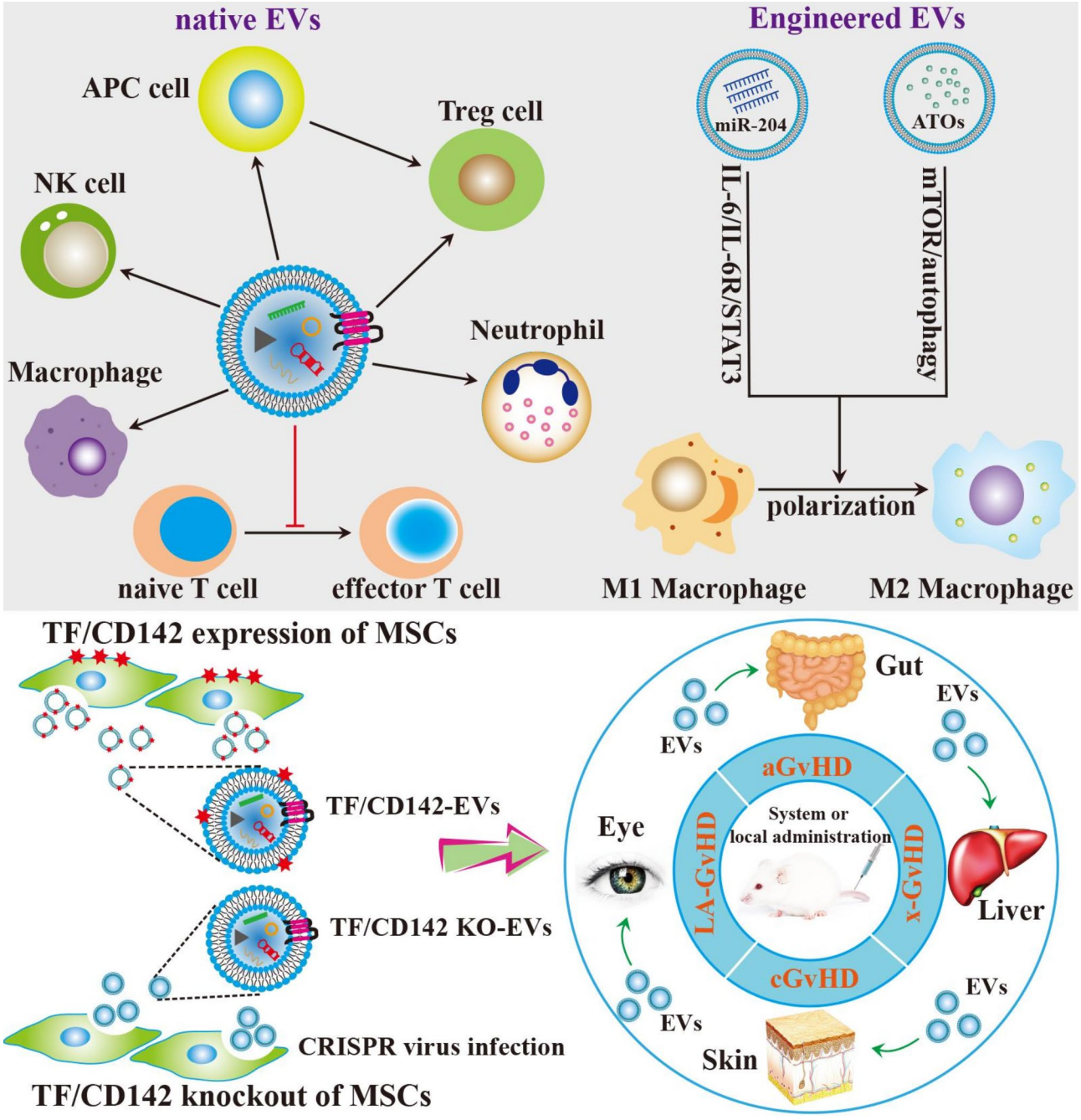

Fig. 4 Application of MSC-EVs in GvHD and its related complications

GvHD results in multi-system damage, with aGvHD primarily impacting target organs such as the gastrointestinal tract, skin, and liver, while cGvHD can affect all organ systems. Research has demonstrated that natural EVs from various sources can modulate the function and homeostasis of immune cells, including T and B cells, through multiple mechanisms of action. This modulation may alleviate the progression of GvHD. Recent studies indicate that engineered EVs can serve as an advanced drug delivery system to enhance the therapeutic efficacy of GvHD while minimizing numerous toxic side effects. MSC-EVs have shown promise in alleviating both acute and chronic forms of GVHD. However, immunogenic proteins and elevated coagulation factors present on the surface of these EVs may compromise their effectiveness in intervening against GvHD. Further research is essential to address these potential challenges associated with EVs in treating GvHD. EVs, Extracellular vesicles; APC cell, Antigen presenting cell; NK cell, Natural killer cell; Treg cell, Regulatory T cell; MSC/MSCs, Mesenchymal Stromal/Stem Cells; M1 and M2, M1 and M2 macrophages; miR-204, miRNA-204; ATOs, Arsenic trioxides; IL-6, Interleukin 6; GvHD, Graft-versus-host disease; aGvHD, Acute GvHD; cGvHD, Chronic GvHD; LA GvHD, Late-onset aGvHD; HRaGvHD, High-Risk aGvHD; SRaGvHD, Steroid-refractory aGvHD; x-GvHD, Human-into-mouse xenogeneic GvHD; TF/CD142, Tissue factor; KO, Knockout; CRISPR, Clustered regularly interspaced short palindromic repeats

an immunomodulatory role in inhibiting the development of autoimmune disorders such as GvHD. Kavian et al. [95]. found that applying ATOs can improve the clinical symptoms of scleroderma GvHD mice. At the same time, Liu et al. [96]. also found that ATOs can transfer macrophages to the M2 state to prolong the survival of aGVHD mice. Other research reports also show that ATOs are expected to be a new method for the treatment of immune disorders, such as experimental colitis and autoimmune encephalomyelitis, rheumatoid arthritis, and systemic lupus erythematosus [97–100], indicating that ATOs may be expected to be a new method for the treatment of GvHD. However, their wide clinical use is limited due to significant toxicity. Therefore, the use of hucMSC-EVs to deliver ATOs cannot only reduce their toxicity but also improve the therapeutic effect of GvHD. Su et al. [29]. prepared ATOs-loaded hucMSC-EVs to intervene in aGvHD. In vitro and in vivo results indicated that ATOs-hucMSC-EVs mainly reduced the severity of aGvHD by regulating the mTOR autophagy signal to reset inflammatory macrophages to the M2 phenotype without weakening the activity of GVL response. In another study, Zhou et al. [78]. found that miR-204 rich in MSC-EVs could effectively relieve the clinical symptoms of GvHD-related DES in both preclinical and prospective clinical trials. Subsequently, to achieve more efficient therapeutic efficacy, the authors constructed an L929 cell line with miR-204 precursor overexpression by gene manipulation and isolated L929-EVs overexpressing miR-204, significantly weakening dry eye symptoms. Establishing this engineered EV therapeutic platform also brought new ideas and hope for treating complex GvHD-related DES.

BMMSCs have been found to regulate T cell homeostasis in vivo, thus protecting patients after allo-HSCT from potentially fatal events of GvHD. Epidermal growth factor (EGF) has been reported to induce the expression of miR-21 and AKT, which improves cell survival in vitro. Additionally, the level of miR-21 is positively correlated with Foxp3 expression of Foxp3 (forkhead box P3), which affects the regulation of the natural function of Tregs. Overexpression of miR-21 has been reported to enhance proliferation, invasion, and differentiation damage to BMMSCs. Zhu et al. (81–82) utilized EGF to facilitate the phosphorylation of c-Jun in BMMSCs, thereby promoting the expression miR-21 in BMMSC-EVs. This led to an enhancement of the expression of Foxp3 in regulatory T cells (Tregs), thereby improving the therapeutic effect of BMSCs on aGvHD. In the aGvHD mouse model, EGF significantly increased the therapeutic efficacy of BMMSCs by reducing the expression of IFN-γ and organ damage. The skin is the site most frequently affected by acute and chronic GvHD [101]. Systemic sclerosis (SSc) and sclerodermatous-GvHD (Scl-GvHD) are chronic autoimmune diseases of connective tissue. Recent studies have shown that MSCs are a promising method for treating autoimmune fibrotic skin diseases SSc and Scl-GvHD. MSCs and their EVs play a crucial role in rebalancing immune and inflammatory disorders, resisting oxidative stress, and inhibiting overly activated fibrosis [102].

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells, which can play a decisive immunosuppressive role in autoimmune response diseases, including aGvHD, and some miRNAs are involved in the pathophysiological process of GvHD. EVs secreted by MSCs stimulated by TGF-β combined with IFN-γ have stronger immunosuppressive activity. They can promote monocyte transformation into Tregs more effectively, among which IDO may be a key mediator [103]. In addition, Wang et al. [104]. found that apoptotic bodies released by IFN-γ/TGF-β1-stimulated apoptotic MSCs significantly inhibited the proliferation of T cells, increased the number of Tregs, maintained the activity of immunoregulatory T cells, and reduced the expression of pro-inflammatory T cells. These research discoveries suggest that the constituents derived from MSCs induced by various stimulating molecules (such as molecules like EVs and apoptotic bodies) likewise offer novel strategies for treating of GvHD and other immune-related disorders.

Studies have shown that the therapeutic MSCs used for therapeutic purposes, as well as EVs secreted by host vascular cells, and these EVs themselves, express varying levels of high procoagulant tissue factor (TF/CD142) on their surfaces [23, 105–107]. TF/CD142 associated with EVs is a crucial endothelial injury and coagulopathy biomarker. Intravenous infusion of MSC may affect the cell's hematological compatibility and safety [108–110]. Therefore, clinicians must be cautious when handling MSC or EV therapy to prevent potentially fatal thromboembolic events during infusion or blood contact, especially in patients with potential coagulopathy, such as GvHD and COVID-19. The immunogenicity of EVs constitutes a significant factor restricting their future clinical transformation and application [111]. At present, basic research has yet to concentrate entirely on this challenge. Hence, it is particularly urgent to gain an in-depth understanding of the various factors influencing the potential immunogenicity of EVs and the strategies for reducing immune recognition to enhance therapeutic efficacy. Hence, eliminating or reducing the expression of TF/CD142 and other immunogenic genes on the surface of MSCs and their derived EVs through CRISPR (Clustered regularly interspaced short palindromic repeats) technology and different approaches can enhance the biocompatibility of MSC-related products after intravenous infusion. These strategies might serve as effective measures to augment the therapeutic efficacy of MSCs or their derived EVs in treating GvHD.

Discussion and perspectives

In the past few decades, studies have shown that EVs have the potential for clinical translation and application in diagnosing and treating various diseases, including GvHD, which has attracted widespread attention from the medical research and clinical application fields. EVs can be secreted and released by almost all cells and are widely present in cell culture supernatant and various body fluids, such as blood, urine, cerebrospinal fluid, and saliva. The existence of the natural lipid bilayer structure of EVs can protect the internal cargo from the degradation of cytoplasmic enzymes to allow the carrying of various functional molecules such as nucleic acid, protein, lipid, metabolites, and other components stably in the extracellular environment; this unique advantage makes EVs a circulating marker of clinical diagnosis and transformation. Many studies have shown that the number of circulating EVs, membrane protein, and internal nucleic acid molecules are significantly different in the occurrence and development of aGvHD and cGvHD, which further suggests that EVs may be used for the prevention, diagnosis, and prognosis monitoring of GvHD as well as guidance of clinical medication. By revealing the expression profiles of EV proteins and nucleic acids and the secretion levels of EVs in different cell subsets during the occurrence and development of GvHD, it is expected to become a promising biomarker for the prevention, diagnosis, and monitoring of disease progression and patient survival. However, challenges persist with insufficient sample sizes, lag isolation methods, and low EV capture yields in EV-based clinical biomarker studies. To overcome the technical bottleneck of EV separation from small plasma volume and improve the yield of EV capture, the researchers independently developed a novel EV technology platform for automatic acoustic capture and separation [18]. The improvement of this technique holds great promise for the early diagnosis of EV-based clinical biomarkers of GvHD. In recent years, the EV separation technology has continuously improved, especially with the emergence of microfluidic and other technologies, so that the cost of EV separation has reduced, the required sample size has reduced, and the capture yield of EV has continually improved [112]. The translational clinical application of plasma-derived EVs in transplant-related diseases will have a broader scope for application.

EVs have biological functions similar to mother cells and advantages such as low immunogenicity, good biocompatibility, and a greater tendency to reach the injured site. Therefore, in recent years, scientists are gradually trying to use natural EVs, especially MSC-EVs, to treat complex and refractory diseases, including diabetes [113], systemic lupus erythematosus [114], rheumatoid arthritis [115], malignant hematological tumors [116], GvHD [12, 27], and other immune disorders [117]. Studies have shown that fibrosis caused by collective barrier surface regeneration failure and maladaptive repair plays a vital role in the pathogenesis and progression of GvHD. Furthermore, median survival, GvHD clinical score, and histological score improved in mice treated with MSC-EV compared to untreated GvHD mice, and prophylactic treatment with MSC-EV reduced the severity of GvHD and improved survival [118]. In addition to MSCs, EVs derived from various immunosuppressive cells can also inhibit the onset and progression of GvHD. Myeloid-derivative-suppressor cells (MDSCs) suppress the immune response. Some studies have shown that MDSCs can inhibit GvHD by promoting Tregs activity, suggesting that MDSC-EVs may be a key component in alleviating GvHD [26]. Although immunosuppressive therapy could control inflammation associated with aGvHD and cGvHD, damage to organ tissue homeostasis will lead to long-term organ damage and affect patient quality of life [6].

MSCs and their derived EVs improve aGvHD and, as well as GvHD-related complications, by mediating tissue regeneration and repair to reduce disease severity of disease and mortality. Unlike traditional nanomaterials, EVs are gradually regarded as natural drug and gene delivery carriers due to their better biocompatibility, safety, and physical and chemical stability [119]. By loading a variety of bioactive molecules into EVs or performing specific targeted modifications on the surface of EVs, these functional EV modification strategies can not only improve the internal circulation time of drugs, reduce drug clearance rate, and reduce drug toxicities but also achieve accurate targeted delivery of drugs, thereby improving the efficacy of treatment. In recent years, some studies have confirmed that engineered EVs can achieve the purpose of improving the treatment of aGvHD and cGvHD and related complications by delivering nucleic acid or drugs and other therapeutic molecules (29, 78, 81–82), which also brings novel ideas and strategies for the treatment of diseases. Previous studies have shown that human BMMSC-EVs could treat acute steroid-refractory GvHD [25]. However, Madel et al. [120]. found that the efficacy of hBMMSC-EV preparations in alleviating symptoms in advanced mouse GvHD models needs to be more consistent between batches. Therefore, standardized MSC-EV production strategies may not guarantee the production of MSC-EV products with repeatable quality and effective mitigation of GvHD. Before administration, every MSC-EV preparation intended for clinical application must thoroughly evaluate its therapeutic efficacy both in vitro and *in vivo.*

Based on EVs, both therapy and drug delivery have been proven to possess favorable safety and efficacy in preclinical models and early clinical trials. Consequently, EVs are anticipated to become the next-generation

therapeutic and drug delivery systems. However, factors such as the origin and biogenesis of EVs, size, endogenous/exogenous contents, production and storage approaches, dosage, infusion rate, and biomolecular corona significantly influence their immunogenicity [121]. Consequently, it is imperative to formulate methodologies for minimizing the immunogenicity of EVs to expedite clinical progress. In addition, there are still some differences and controversies in the purification and identification, bioactivity determination, and quality control of EVs derived from stem cells, including embryonic stem cells and MSCs, and there still needs to be unified standards. To address these challenges and actively promote the research and application of stem cell-derived EVs in the disease treatment field, the Extracellular Vesicle Research and Application Branch of the Chinese Research Hospital Association enlisted domestic experts and scholars specializing in stem cell-derived EVs. In 2022, for the first time, two national group standards, "Small Extracellular Vesicles Derived from Human Pluripotent Stem Cells" (T/CRHA 002-2021) [122] and "Small Extracellular Vesicles Derived from Human Mesenchymal Stem Cells" (T/CRHA 001-2021) [123], were formulated and published.

Conclusions

Although EVs have broad application potential and advantages in the diagnosis and treatment of diseases, there are still many bottlenecks and challenges in clinical translation and application, including purity and standard extraction, large-scale production and preparation, and long-term in vivo application safety evaluation, still have a long way to go. It is postulated that via the concerted endeavors of scholars specializing in EV research both nationally and internationally, in conjunction with the progressive enhancement of EV separation and purification technology, certain impediments that EVs confront in diagnosis and application will be successively surmounted. Additionally, the application and transformation of stem cell EVs in the therapeutic management of multiple intricate and recalcitrant clinical disorders such as GvHD will also exhibit more promising prospects and application values.

Acknowledgements
Not applicable.

Author contributions
P.W. and Z.W. designed, drafted, wrote, and revised this manuscript. Y.S. participated in editing and revising the paper. P.W., Z.C., M.W., and B.W. participated in the conception and design, financial support, and final approval of the manuscript. All authors read and approved the final manuscript.

Funding
This study was supported by grants from the National Natural Science Foundation of China (No. 82303775); the Natural Science Foundation of Anhui Province (2308085QH273); China Postdoctoral Science Foundation (2023M733398); Natural Science Research Project of Anhui Educational Committee (No. 2022AH050739); Research Fund of Anhui Institute of translational medicine (No. 2022zhyx-C65); The Youth Project of the Research Fund of Anhui Medical University (No. 2023xkj018).

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors consent to publication.

Competing interests
The authors declare no competing interests.

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Received: 16 October 2024 / Accepted: 4 March 2025
Published online: 26 March 2025

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