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Extracellular Vesicles and Lupus Nephritis -:

New insights into pathophysiology and clinical implications

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Abstract

Lupus nephritis (LN) is a major cause for overall morbidity and mortality in patients with systemic lupus erythematosus (SLE), while its pathogenic mechanisms are still not well understood. Extracellular vesicles (EVs) are membrane vesicles that are released from almost all cell types. EVs can be subdivided into exosomes, microvesicles, and apoptotic bodies. Latest studies have shown that EVs can be released during several cellular events, including cell activation, autophagy, and several types of programed cell death, i.e. apoptosis, necroptosis, pyroptosis, and NETosis. Emerging evidence demonstrates that EVs harbor different bioactive molecules, including nucleic acids, proteins, lipids, cytokines, immune complexes (ICs), complements, and other molecules, some of which may contribute to pathogenesis of autoimmune diseases. EVs can serve as novel information shuttle to mediate local autocrine or paracrine signals to nearby cells, and distant endocrine signals to cells located far away. In LN, EVs may have pathogenic effects by transportation of autoantigens or complements, promotion of IC deposition or complement activation, and stimulation of inflammatory responses, renal tissue injury, or microthrombus formation. Additionally, EVs released from kidney cells may serve as specific biomarkers for diagnosis or monitoring of disease activity and therapeutic efficacy. In this review, we will summarize the latest progress about EV generation from basic research, their potential pathologic effects on LN, and their clinical implications. The cutting-edge knowledge about EV research provides insights into novel therapeutic strategy, new tools for diagnosis or prognosis, and evaluation approaches for treatment effectiveness in LN.

Keywords

extracellular vesicles; autoantigen; immune complex; inflammation; lupus nephritis

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1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by presence of autoantibodies, immune complexes (ICs) and complement deposition, and the relevant autoimmune inflammation in different organs/tissues, including kidney [1]. Lupus nephritis (LN) affects 30–60% of adults and up to 70% of children with SLE [2]. LN results in gradual decline of kidney function and renal failure and is a major cause of morbidity and mortality in SLE patients.

Extracellular vesicles (EVs) are a heterogeneous group of membrane vesicles released from cells to extracellular space. Almost all mammalian cell types, even lower eukaryotes, prokaryotes, and plant cells can release EVs. This fact indicates that EV-mediated cell signaling might be essential mechanisms for intercellular communication that emerged in early biological evolution of all living organisms [3]. EVs have been found from nearly all kinds of body fluids and solid organs/tissues, and involved in not only normal physiological events [4, 5], such as immune surveillance, cell-to-cell communication, inflammation and blood coagulation, but also abnormal pathological conditions in various human diseases [6, 7], including autoimmune and cardio-metabolic diseases, as well as cancer development and metastasis.

Cell death is a natural biological process that occurs under both physiological and pathological conditions. To date, over ten types of programmed cell death have been identified [8]. Apoptosis is the most studied type of programmed cell death. Both exosomes and membrane microvesicles can be released in cells undergoing apoptosis, apoptotic bodies are the corpse of apoptotic cells that are formed in the end stage of apoptosis [9]. Recent studies demonstrated that several other types of programmed cell death, i.e. necroptosis, pyroptosis, and NETosis can also cause release of membrane microvesicles [10–14]. Cell death is critical for maintaining homeostasis. Excessive cell death and/or defective clearance of dead cells and their released EVs may break immune tolerance and trigger immune and autoimmune responses in the body [15, 16].

Recent evidence indicates the involvement of EVs in pathogenesis and clinical complications of SLE and LN [17]. Autoantigens generated during apoptosis are clustered, and redistributed into the membrane surface of EVs or apoptotic bodies [18]. The apoptotic EV-associated autoantigens may trigger B cells for adaptive immune response in SLE [19]. EV-associated autoantigens form immune complexes (ICs) with autoantibodies, resulting in formation of EV-ICs [20, 21] which could thus be regarded as large ICs with capacity for deposition in organs/tissues, including kidney [17, 22]. Immune electron microscopy studies have provided the evidence of co-localization of glomerular deposited ICs with microvesicles and galectin-3-binding protein (G3BP) in LN [22]. EV-ICs may activate complements and contribute to endothelial activation, tissue damage, cellular proliferation, and proinflammatory responses in the pathogenesis of LN [23, 24]. In this review, we will summarize the latest progress and recent advances in EV research, potential involvement of EVs in pathogenesis of LN, as well as their clinical implications.

2. Recent progress in generation of Extracellular Vesicles

Based on their origin and physical/biological features, EVs can be subdivided into three main classes , including exosomes (<100 nm), microvesicles (MVs) (<1 μm) and apoptotic bodies (1–5μm) [5]. Exosomes are generated by exocytosis of endosomal derived intracellular membrane vesicles to the extracellular space. Exosomes have been reported to contribute to many aspects of normal physiology, pathological conditions and human diseases [25]. In contrast, microvesicles (MVs, also called microparticles) are larger membrane vesicles derived from cell plasma membrane surface. Since MVs bud from cell membrane surface, the cell membrane-associated molecules are known to be released with MVs [6, 26]. In addition, studies from our and other groups found that cytosolic molecules and even nuclear molecules can also be associated with MV membranes [27–30] or encapsulated in the lumen of MVs [31]. Apoptotic bodies are the largest size membrane vesicles that are generated during apoptosis and can carry nuclear fragments and mitochondria, therefore are important in autoimmune diseases [9]. Here we have briefly illustrated the recent progress in cell death related EV generation (Figs. 1 and 2).

During apoptosis, procaspase 3 can be cleaved and become active caspase 3, which can activate Rho-associated protein kinase 1 (ROCK1) that is involved in apoptotic membrane blebbing through regulation of actin–myosin contraction [9]. In addition to the release of MVs and apoptotic bodies from apoptotic cells, Park et al reported that a fraction of exosome-like vesicles can also be released from apoptotic cells [32]. Their biogenesis was completely dependent on cellular sphingosine-1-phosphate (S1P)/S1P receptors (S1PRs) signaling [32]. Apoptosis, thus, can release full spectrum of EVs, from very small exosomes, medium sized microvesicles, to large apoptotic bodies. Apoptosis has long been known to be involved in SLE in numerous studies [33]. With the context of clearance deficiency of apoptotic debris, the remnants of apoptotic cells cumulatively challenge immune tolerance and continuously induce autoimmune and inflammatory responses in patients with SLE [34].

Pyroptosis has been shown to be regulated by caspase-1-dependent canonical inflammasome pathway and caspase-1-independent non-canonical inflammasome pathway [35, 36]. Caspase-1-independent pyroptosis is executed by caspase-11 in mice, while regulated by caspase-4/5 in human [35, 36]. Latest studies found that pyroptotic monocytes can release a heterogeneous population of EVs [11]. These EVs encapsulated Gasdermin D (GSDMD), caspases-1 [12] and Fas-associated death domain (FADD) [37]. Furthermore, MVs that contain both cleaved GSDMD and active caspase 1 could induce vascular endothelial cell injury [12].

Furthermore, necroptosis is a form of programmed cell death that critically depends on receptor-interacting serine-threonine kinase 3 (RIPK3) and mixed lineage kinase domain-like (MLKL) and generally manifests with morphological features of necrosis [38]. Necroptotic cells can release EVs and mitochondria to extracellular space [14, 39, 40], under the regulation by RIPK3 and MLKL [14]. Furthermore, release of phospho-MLKL in EVs can downregulate the cellular content of phospho-MLKL in their parental cells, thereby protecting cells from necrotic cell death [14] .Very recently, Thiam et al reported that neutrophils undergoing NETosis can also release membrane MVs before neutrophil

extracellular trap (NET) formation [13]. Although the underlying cellular mechanism is unclear, cytoskeletal rearrangement might be involved [13]. In addition, a recent study also reported that autophagy can regulate exosomal release of prions in neuronal cells [41]. Exosomes arise from endosomal-derived multivesicular bodies, and crosstalk between autophagy and the endo/exo-somal vesicular trafficking pathways might be involved in exosome biogenesis during autophagy [42, 43].

In addition to apoptosis, the above discussed other types of programmed cell death have also been associated with pathogenesis of SLE and LN [44]. For instance, activation of necroptosis pathway and the RIPK3 dependent NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome pathway in podocytes have been shown to be involved in LN pathogenesis [45]. B cells from SLE patients shows high expression levels of necroptosis-related genes [46]. Furthermore, autophagosomes have been observed in podocytes in mice and human, and increased autophagy can exert a cytoprotective impact on podocyte injury [47]. Extensive EV research indicates that not only the cells that undergo cell death, but also their released subcellular EVs, can contribute to autoimmune responses and lupus development through various pathogenic functions. However, there is only limited information in the literature regarding the involvement of pyroptosis [48, 49] and necroptosis [45, 46] in lupus. While, there is clue for the potential link between these two types of cell death and SLE, and EVs may be involved in the pathogenesis of LN.

3. Potential pathologic effects of Extracellular Vesicles on Lupus Nephritis

The central paradigm of SLE is loss of immune tolerance to the sustained autoantibody production, while LN is a form of glomerulonephritis with deposition of ICs and complements [50]. In SLE, impaired macrophage phagocytic capacity results in defective clearance of apoptotic bodies, accumulation of autoantigens, and sustained production of autoantibodies with elevated formation of ICs [51, 52]. Nucleic acid-containing ICs drive inflammation through activation of Fcγ receptors (FcγR), complements, and the type I interferon pathway by engaging toll-like receptors (TLRs) [53, 54]. In addition, deposition of ICs and complements in kidney results in endothelial damage, and consequent microthrombi formation, as well as renal tissue damage and cellular proliferation in kidneys of LN patients [24, 53, 55].

EVs have been shown to be involved in pathogenesis of SLE and LN, through different mechanisms. Based on the current understanding of the lupus pathogenesis, we have summarized the potential pathologic effects of EVs on LN (with illustration Fig. 3) in the following aspects: 1) EVs may function as sources of extracellular autoantigens; 2) EV-associated autoantigens may stimulate B cells to generate autoantibodies, and then form EV-ICs, which may deposit in glomeruli; In addition, EVs may 3) either carry complement components or serve as a platform to activate complement system, and 4) cause renal tissue damage; Furthermore, EVs also contribute to 5) autoinflammatory responses and proinflammatory cytokine production, 6) prothrombotic conditions by carrying procoagulant properties. In addition, we also summarized the published clinical studies regarding the involvement of EVs in LN (Table 1).

3.1 EVs as the source of autoantigens

Accumulation of autoantigens from dying and dead cells is an important feature in SLE [56]. Certain molecules on nucleus, cytoplasmic, and even plasma membrane, can be clustered as autoantigens in EVs during various cellular events. Nuclear autoantigens are typically not accessible to the immune system [6]. Studies from our and other groups have demonstrated the redistribution of nuclear molecules into cytoplasm and plasma membrane where they may release with MVs during membrane budding or blebbing [29, 57, 58]. It has long been known that nuclear autoantigens (nucleosomal DNA, Ro, La) clustered on the membrane surface in the late stage apoptotic cells [58]. Other nuclear autoantigens, including histone nuclear proteins, lamin B1, and non-histone nuclear protein (like high mobility group box 1 (HMGB1)), can also be released with MVs [29, 59, 60]. In addition, cells undergoing pyroptosis and necroptosis could also release EVs with nuclear or mitochondrial molecules [11, 40] which may also serve as autoantigens.

Furthermore, cytoplasmic molecules may also be loaded into EVs, including mitochondria [61], proteinase 3 (PR3) [62] and myeloperoxidase (MPO) [63, 64]. Neutrophil PR3 or MPO are known to serve as autoantigens in the development of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis [26]. Both PR3 [62] and MPO [64] have been found to be associated with EVs. This suggests these EV-associated PR3 and MPO may serve as autoantigens that trigger production of the corresponding autoantibodies in LN patients. In line with this hypothesis, Turner-Stokes et al reported that patients with LN have positive ANCA serology [65].

It has long been known that negatively charged plasma membrane PS regulates cellular localization of positively charged proteins [66]. The net positive charge of nuclear HMGB1[29] and cytoplasmic MPO and PR3 [67] provide the chemical basis for their binding with negatively charged PS on plasma membrane [29] during molecule redistribution in activated or apoptotic cells, and thus release with MVs during cell membrane budding. Translocation and association with phospholipids on plasma membrane also contribute to neoantigen properties of those intracellular molecules on EVs [68]. In NETotic cells, histone hypercitrullination modification is the key for chromatin de-condensation and NET formation [69]. Studies have reported that NETs can be the source of citrullinated histone autoantigens that contribute to development of autoimmune diseases [70]. A recent study have found that NETotic neutrophils can also release EVs, however these EVs do not contain nuclear DNA [13]. It does not know if the NETotic EVs also associate with citrullination-modified autoantigens.

In normal healthy cells, the nuclear and cytosolic autoantigenic molecules are located within nucleus or cytoplasm which are surrounded by the nuclear envelope or plasma membrane respectively. Thus, the nuclear and cytosolic autoantigenic molecules cannot be accessed by the immune system [57]. Autoreactive inflammatory responses in lupus require exposure and delivery of autoantigens to antigen presenting cells (APCs) in a proinflammatory context. During apoptosis, membrane translocation of nuclear and cytosolic autoantigens can enhance their exposure and accessibility by APCs [71]. Thus, the self-antigens from nucleus, cytoplasm, or mitochondria may expose on the membrane surface of EVs during the redistribution of autoantigenic molecules and EV budding process, making

them accessible by APCs of immune surveillance system. Besides, impaired phagocytic clearance of apoptotic corps by macrophages in SLE patients [52] may enhance extracellular accumulation of EVs with autoantigens [72].

Interestingly, EV-associated autoantigens have potential to stimulate autoreactive inflammatory responses. Ullal et al reported that MVs with DNA and nucleosomal molecules can bind anti-DNA and anti-nucleosomal antibodies, and lupus plasma contains MV-ICs [73]. Binding of nucleosome-containing EVs to B cells can stimulate B proliferation and induce antibody secretion [59]. Furthermore, artificially encapsulating mammalian DNA by transfection reagent (lipofectin) has been shown to increase the immunostimulatory effects *in vitro* and immune responses in cultured cells [74]. This study suggests that EVs may serve as the endogenous source of encapsulated DNA and exert immunostimulatory properties. All in all, the above discussed findings indicate that EVs may be potential sources of autoantigens by carrying autoantigenic molecules from nucleus, cytoplasm, or cell membrane, therefore contributing to autoimmune responses in lupus.

3.2 EVs and immune complex deposition in renal glomeruli

LN results from glomerular IC deposition and the consequent chronic inflammatory responses. Studies have detected the EV-containing immune complexes (EV-ICs) in SLE patients [21, 22, 75], therefore one may expect to see potential role of EV-ICs in pathogenesis of LN. Fujigaki and colleagues have reported deposition of ICs, complements, and EVs in glomerular basement membrane (GBM) and the nearby epithelial cells in glomerulonephritis rats [76]. G3BP is a multifunctional glycoprotein which has soluble and cell-associated forms [77]. G3BP can be associated with MVs, G3BP-positive MVs and IC-associated MVs are significantly increased in SLE patients [78]. Most interestingly, electron microscopy analysis found the co-localization of G3BP with in vivo-bound IgG in the glomeruli of kidney biopsies from LN patients [22]. Since G3BP is known to mediate cell-cell or cell-matrix adhesion, G3BP-positive MVs may facilitate the deposition of these large EV-ICs in the GBM leading to formation of electron dense structures and nephritis [17].

The GBM is the central and non-cellular layer of the glomerular filtration barrier which is composed primarily of four types of extracellular matrix (ECM) macromoleculelaminin-521, type IV collagen, heparan sulphate (HS) proteoglycan (HSPG), and nidogen [79]. HS provides the molecular source of negative charges in the GBM [79]. Gallo et al found that electrostatic interactions between cationic status of ICs and the fixed anionic sites on GBM may be an important factor in glomerular trapping [80]. Even a small portion of cationic antibodies in ICs may affect their net electrostatic condition [81] resulting in the positive charge of ICs that is sufficient to mediate their deposition in GBM of glomeruli [80, 81]. As an important component of nuclear DNA, positively charged histone has been reported to mediate glomerular deposition of small size DNA and anti-DNA complex[82]. The positively charged histone-associated MVs [83] may mediate the glomerular deposition of EV-ICs by binding to the negatively charged HSPG on GBM, and contribute to development of LN. Furthermore, fibronectin is an ubiquitously distributed glycoprotein in the ECM, and it has been detected on the membrane surface of EVs in various studies [84]. As fibronectin

has specific binding sites for HSPGs, it was reported to mediate EV-cell interactions by serving as a bridge between EV-HSPGs and cell membrane-HSPGs in ECM [84]. Elevated levels of plasma fibronectin have been reported in SLE patients [85] and anti-fibronectin antibodies are also detected in portion of SLE patients [86]. However, it's not clear yet if fibronectin-positive EVs may potentially mediate EV-ICs deposition in glomeruli through molecular interaction between fibronectin and HSPG in GBM.

Furthermore, MVs provide adhesion and costimulatory molecules that may contribute to the ICs deposition when MVs bind to various cells, e.g., to endothelial cells (ECs) in kidney glomeruli. This may explain the presence of autoantibodies at sites where the specific epitopes are absent [75, 87]. In addition, it is not known whether EV-ICs are more difficult to be cleared as compared to conventional ICs. Although PS is a commonly used MV surface marker, portion of MVs are PS negative. Given that PS exposure serves as "eat-me" signal and mediates MVs clearance by macrophages, therefore PS-negative EV-ICs might not be efficiently cleared [21].

3.3 EVs carry or activate complements

Complement system is part of the innate immune system and is involved in the development of LN [24]. In pathologic studies, complements C5b-9 complexes have been detected in sub-epithelial immune deposits and epithelial MVs under electron microscopy in glomerulonephritis rats [76]. Co-presence of EVs and complements in nephritic glomeruli indicates a potential link between the two during immune responses in nephritis, including LN. Ostergaard and colleagues reported that circulating EVs in SLE are enriched with ICs and complements, particularly the complement proteins from the classical pathway [78]. As the common feature of complement activation pathways, conversion of C3 to C3b can activate a stable thioester bond, leading to the covalent attachment of C3b to cell-surface [88], and the downstream cascade molecule activation, as well as formation of MAC on the cell surface that make them possible to be associated with MVs during cell membrane budding process [89]. To assure cell survival and recovery from complement attack, cells may release a subgroup of EVs bearing complement molecules, especially MAC, as a self-protective mechanism to clear complements actively [23]. Such complement-associated EV shedding may result in formation of EVs binding with C3 fragments [90], C9 [90], MAC [91] and complement regulators, like complement receptor 1 (CR1/CD35) [92], CD55, CD59 [93].

Complement activation contributes to a variety of vascular and inflammatory disease states, including LN. Recent studies also demonstrate that EVs may serve as a platform for activation of complement system [23]. ICs are known to be the major activator of classical pathways. Nielsen and colleagues reported that elevated level of immunoglobulins and complements in EVs are correlated with autoantibodies and complement activation [75]. Furthermore, binding of C1q to apoptotic bodies induces activation of the classical complement pathway [94]. Therefore, deposition of ICs, complements, and EVs in GBM in rats with glomerulonephritis detected by electron microscopy [76] suggest their potential interaction in kidney. Interestingly, even without antigen harboring, the phospholipid components of EVs may make complements binding to lipid membranes

through electrostatic interactions with surface charge of the phospholipids on EVs [95], and then subsequently leading to deposition and activation of C4 and C3 on EV surface [96]. Therefore, erythrocyte-derived MVs have been reported to fix C1q, following by subsequent activation of the classical pathway complement with binding of C3 fragments [97]. In addition, lipid components, like PS, on EVs could also be activator of complement system through the alternative pathway [98].

On the other hand, APCs-derived exosomes binding with complement regulators, i.e. CD55 and CD59, can functionally inhibit complement activation *in vitro* by inhibiting complement-mediated lysis [99]. Furthermore, CRl-loaded EVs released by glomerular podocytes [92] may reduce complement-mediated damage by inactivation of C3b in kidney [100]. It's unclear whether delivering the EV-associated complement regulators/inhibitors can be protective to lupus. However, this could be a new avenue for investigation and development of novel therapeutic strategy for LN treatment. These are still many unanswered questions need to be explored in the future.

3.4 Involvement of EVs in tissue damage in lupus nephritis

As discussed above, deposition of ICs in glomeruli of LN can activate complements that initiate cell damage, generate chemoattractant factors, and activate immune cells (i.e., neutrophils, monocyte, dendritic cells) through FcγR, leading to release of reactive oxygen species (ROS) and proinflammatory cytokines, thus consequently amplifying local immune responses in lupus kidney [53]. In addition, EV-associated pro-inflammatory molecules may directly act on target cells and cause the release of pro-inflammatory cytokines [101–104], thus contribute to inflammation and tissue damage. The inflammatory milieu, induced by EVs and EV-ICs, may induce podocyte injury and foot process effacement, proliferation of mesangial and parietal epithelial cells, leading to leakage of plasma and proteinuria and glomerular dysfunction [51, 105, 106].

Increased circulating ECs and endothelial EVs have been detected in patients with LN [107] or SLE [108], reflecting microvascular injury in these patients. MPO is a positive charged molecule that can easily bind with the negatively charged structures [109], including glomerular ECs, thus may induce endothelial damage [110] and contribute to glomerulonephritis [109]. O'sullivan et al found that many damaged ECs were MPO positive [109]. Regarding the mechanisms by which MPO may be internalized by ECs , it has been reported that β2-integrin-mediated cell-cell contact between neutrophils and ECs has been reported for direct transfer of MPO from neutrophils to ECs [111]. On the other hand, the *in vitro* experiments have demonstrated that extracellular MPO can be translocated and internalized into ECs and epithelial cells [112]. In addition, neutrophil-derived MPO-positive EVs have been shown to induce vascular endothelial cell damage [63]. Serum levels of anti-MPO have been associated with future LN [113]. The glomerular endothelial damage may trigger vascular microthrombosis [50] that could mechanically obstruct glomerular capillaries, diminishing the blood supply to glomeruli and renal tubules, thereby further causing chronic hypoxic/ischemic injuries on the affected glomeruli and tubules during the development of LN [114].

3.5 EVs and autoimmune inflammation in Lupus Nephritis

Increasing evidence demonstrates that EVs have been associated with autoimmune inflammatory responses in LN. The defective clearance capability of macrophages in SLE [72] results in accumulation of dead cells and their released EVs may contribute to autoimmune inflammation in LN. Cargoes of EVs from nucleus, cytoplasm, or cell membrane of various cellular origins may contribute to pro-inflammatory milieu through EV-associated proinflammatory properties. A recent study demonstrated that many cytokines are released in EV-encapsulated forms *in vitro*, *ex vivo*, and *in vivo*, and these EV-encapsulated cytokines are capable of eliciting biological effects upon contact with target cells [31]. MVs released from monocytes-stimulated by lipopolysaccharide contain IL-1β and inflammasome [115]. These monocytic MVs can also activate ECs to express adhesion molecules, thus further promoting recruitment of inflammatory cells [115]. In turn, EVs shed from activated ECs contain high levels of adhesion molecules, i.e. E-selectin, which can mediate adhesion of monocytes to ECs *in vitro* [116]. Additionally, we have reported that nuclear HMGB1 can be translocated to plasma membrane and release with EVs to extracellular space [29]. Elevated levels of HMGB1-positive EVs have been found in circulation and urine in SLE patients with LN [117]. Extracellular HMGB1 is an endogenous prototypic damage-associated molecular pattern (DAMP) molecule and has been implicated in several inflammatory disorders [118]. Monocytes and macrophages can recognize HMGB1 through TLR4 receptor, resulting in production of pro-inflammatory cytokines [119].

In addition, EVs may also propagate inflammation indirectly by stimulating other cells to produce proinflammatory materials. EVs or EV-ICs have been reported to activate monocytes to produce pro-inflammatory cytokines, i.e. TNF-α, IL-8, IL-6, and IFN-α [20]. It has been reported that platelet-derived MVs contain both IL-1α and IL-1β that can induce IL-6 and IL-8 production by synoviocytes, indicating their pro-inflammatory potential [120]. Similarly, platelet EV-ICs are highly pro-inflammatory and can elicit leukotriene production from neutrophils [121]. Additionally, EV-associated miRNAs may regulate inflammatory cytokine production in target cells [101]. Recently, Salvi et al. demonstrated that circulating exosome-associated miRNAs from SLE patients can trigger human primary plasmacytoid dendritic cells (pDCs) to produce IFNα, TNF-α and IL-6 through TLR7-dependent activation [122]. Taken together, EVs may contribute to a proinflammatory milieu and autoimmune inflammation in LN either directly with their associated proinflammatory components or indirectly by triggering other cells to produce proinflammatory cytokines or proinflammatory materials.

3.6 EVs and glomerular microthrombosis in Lupus Nephritis

Numerous studies have revealed that presence of microvascular lesions and microthrombus formation could adversely affect the course of renal disease [123]. Prothrombotic function is the first described pathological feature [124] and the mostly studied classical function of EVs [27, 28] . Our own studies have shown that EVs have pro-coagulant properties [27, 28], particularly the monocyte-derived EVs (mEVs) with both tissue factors (TF) and PS exposed on the membrane surface [27, 28]. During the EV formation process, the plasma membrane asymmetry is lost due to membrane and cytoskeleton rearrangements, resulting

in membrane exposure of anionic PS on the membrane surface of EVs [125]. In monocytes, the membrane expressed TF can also be released with mEVs during membrane budding [27, 28]. Co-presence of PS with TF on EVs enhances the procoagulant potential as TF can trigger extrinsic coagulation cascade, while the negatively charged PS provides an ideal surface for assembly of coagulation factors [26, 126]. Furthermore, the endothelial derived EVs that express TF might also promote microthrombus formation [127] as well. Interestingly, platelet- and erythrocyte-derived MVs can also initiate thrombin formation in a FXII-dependent manner through intrinsic pathway [128].

Among various renal vasculopathies, thrombotic microangiopathy (TMA), in which complement may be involved [129], represents the most severe vascular manifestations with highest mortality rate and results in renal microvascular thrombosis due to vascular endothelial injury [130]. As described above, EVs may carry complement and serve as a platform for activation of complement system with EV-associated components. Lood et al reported that complement deposition on platelets may be related to thrombosis in SLE patients [131]. Another study with complement C3 or C5 knockout mice have demonstrated the specific roles of C3 in platelet activation and complement-dependent membrane perturbations, leading to prothrombotic TF activation on myeloid cells [132]. Furthermore, it has long been known that MAC can induce vesiculation in endothelial cell membrane and expose catalytic surface for prothrombinase enzyme complex [133]. Additionally, complement activation has been associated with thrombosis in antiphospholipid (aPL) syndrome [134], a common syndrome of SLE. The majority of pathogenic aPL antibodies retain their reactivity with membrane lipids, and induce NADPH-oxidase (NOX)-dependent proinflammatory signaling and TF activation, with assistance of complements [134]. Thus, TF primes monocytes for thrombosis amplification through a crosstalk between complement activation and coagulation signaling [134], EVs may be able to involve in these process with their-associated TF or complements, although there is no direct study has been reported yet.

According to Virchow’s triad, the pathophysiologic thrombosis is achieved by interplay of abnormalities in blood composition, vessel wall injury, and blood flow disturbance [135]. Elevated thrombogenicity by pro-coagulant EVs, while abnormal vessel wall by EV-associated endothelial dysfunction or damage [6, 63, 136], thus EVs may contribute to thrombosis by affecting at least two elements of Virchow’s triad. It has long been known that inflammation and thrombosis are related [137], EVs may play a central role in inflammation-related hypercoagulability state, thus contributing to LN.

In addition to the prothrombotic properties of EVs, they may also carry proteins with coagulation inhibitory properties, such as TF pathway inhibitor [138], protein C [139]. This fact indicates the complexity of contributions of EVs to microthrombus formation. The balance between anti- and pro-thrombotic properties of EVs as well as their contribution to LN still need to be further explored.

4. Potential clinical implications of EVs on Lupus Nephritis

The current available therapeutic strategies in LN primary management still rely heavily on non-specific immunosuppressive treatment. Targeted therapies may offer promising potential

for improved therapeutic efficacy with fewer side-effects [140–143]. Although the current clinical trials for emerging therapeutic strategies mainly target on B cells, T cells, or IFNs for lupus treatment, the heterogeneity features of lupus make it very difficult to set ideal targets. As discussed above, recent progress indicates that EVs released by infiltrating immune cells or renal resident cells convey in circulation or spread in local renal tissues by interaction with either innate or adaptive immune cells, involving in almost all aspects of pathogenesis in SLE or LN. Regulation of EV generation or interfering their pathologic involvements in LN may provide insights into novel therapeutic strategies for SLE and LN treatment.

Through selectively packaging process, EVs harbor or enclose specific DNA, RNA, or proteins thus mediate intercellular communications between neighboring cells or distant cells [144]. This newly discovered approach for old cellular mechanism of intercellular communications could be novel therapeutic targets in various human diseases, including lupus. Cantaluppi et al have reported that EVs derived from endothelial progenitor cells, containing different mRNAs coding for several anti-apoptotic signals and for complement inhibitors, demonstrated protective effects in experimental complement-mediated glomerulonephritis [145]. Other beneficial effects of EVs may also attribute to modulation of fibrosis, tubular and glomerular damage, and angiogenesis by EV-associated contents [146], i.e. mesenchymal stem cell-derived EVs for renal repairment [147]. Furthermore, engineered exosome vector fused with the targeting peptide RVG (rabies viral glycoprotein peptide) has been used to direct exosomes to kidney mediated by acetylcholine receptor [148]. Interestingly, the engineered EVs containing specific contents selectively target to kidney cells for treatment of kidney diseases, including LN, can be expected for future novel treatment. All of the above information shines the light for new era of EV application as emerging therapeutic methods for human diseases, including LN.

Based on the above discussed, EVs contain the information that reflects pathophysiological conditions or disease severity. Therefore, analysis of EVs in urine or plasma may serve as non-invasive “liquid biopsy” [149, 150] approaches for diagnosis and prognosis of LN, in contrast to the classical invasive renal biopsy. Changes in levels of urinary podocyte EVs may reflect ultrastructural pathologic changes of podocytes in patients with active LN in advance of classical albuminuria/nephrin [151]. From the perspective point of view, EVs may have predictive value for monitoring the disease severity and prognosis for future outcomes, although a lot of work needs to be done before the validated methodologies can be established. Furthermore, high levels of cell free DNA (cfDNA) in SLE patients were reported early in 1966 [152]. Since then, there has been increasing interest in applying cfDNA as a potential biomarker for diagnostic purpose of various human diseases, including lupus. Overall, lupus patients show elevated levels of cfDNA that fluctuate concomitantly with disease activity, inflammatory markers and to some extent with therapeutic interventions [153]. A recent study has reported that large portion of plasma cfDNA is associated with EVs [154]. Therefore, detection of EV-associated cell free DNA might be useful for patients with SLE or LN.

Non-coding RNAs (ncRNAs), including long non-coding RNA (lncRNA), circular RNA (circRNA), and micro RNA (miRNA), has been shown to be associated with EVs [155–157].

Recent studies have revealed that the ncRNAs play an important role in the pathogenesis of SLE and/or LN [158–160]. Although studies have shown that EVs can carry ncRNAs, and ncRNAs have been shown to be involved in lupus pathogenesis, while there are no published papers that directly study the presence of lncRNA and circRNA in EVs in SLE patients. In the perspective point of view, this novel area is worthy to be investigated. There are several published papers that have studied EV-associated miRNAs in the context of lupus. Firstly, changes of miRNAs in urinary EVs have been reported in LN [161]. These findings demonstrated that increased miR-146a [162], miR-26a [163] and down-regulation of let-7a, miR-21 [164], miR-29c [165] in urinary exosomes could discriminate the presence of LN. Secondly, changes of miRNA level in EVs could also reflect the injury of podocytes [105] and early renal fibrosis in LN [165]. Thirdly, levels of let-7a and miR-21 in urinary exosomes, which were down-regulated during disease flare, were elevated after complete course of treatment[164]. These findings indicate that miRNAs in EVs can be served as potential biomarkers for disease diagnosis and monitoring. More importantly, uptake of urinary exosomes with high levels of miR-31, miR-107, and miR-135b-5p by mesangial cells regulate LN renal recovery by HIF1A (hypoxia inducible factor 1-alpha) inhibition [166]. In addition, circulating exosome-associated miRNAs from SLE patients have been shown to trigger human primary plasmacytoid dendritic cells (pDCs) to produce IFNα, TNF-α and IL-6 through TLR7-dependent activation [122].

Levels of cfDNA and miRNAs in EVs may have great potential to serve as biomarkers in LN. However, it requires a rigorous evaluation of cfDNA and miRNAs in longitudinal studies with large cohorts of patients and careful comparison with existing inflammatory and clinical markers of disease. The relatively simple and practically applicable assay kits for detection of urinary EVs and their-associated specific biomarkers are expected to be developed for the “precision medicine” for future improvement of clinical management of LN instead of the potentially hazardous and expensive renal biopsy. These efforts are also important for optimization of treatment efficacy for LN patients.

5. Conclusions

In this review, we have summarized the latest progress in cellular mechanisms about the EV generation in several novel types of programmed cell death in addition to apoptosis. The research progress on EV generation may provide insights into new understanding regarding the involvement of these novel types of programmed cell death in SLE and LN. The article also summarized the current literatures and give our interpretation based on our experiences in EV research for over decade. In the article, we summarized the potential pathogenic effects of EVs on several aspects of pathogenesis of LN, i.e. EVs serving as sources of autoantigens, mediating immune complexes renal deposition, harboring or activating complements, inducing renal tissue damage, as well as promoting inflammatory responses and microthrombosis, thus leading to sustained autoimmune inflammation in kidney. The cutting-edge knowledge summarized in the article may provide the insight into novel therapeutic avenues over the current targets of the clinical trials for new treatment in lupus. Furthermore, the current progress in EV research may also be helpful to establish new tools for diagnosis biomarkers, monitoring, prognosis of disease activity, and therapeutic efficacy in patients with autoimmune diseases, including SLE and LN. Whether therapeutic

or diagnostic strategies towards EVs can be translated into future clinical practice in LN patients are still not fully understood. A lot of efforts are still needed to find out potential targets on EVs for their crucial roles on LN pathogenesis. And the long-term follow-up studies are also needed to evaluate the possible roles of EVs as clinical biomarkers in LN. To date, further studies are necessary to explore terra incognita about EV generation, regulation and the involvement in human diseases.

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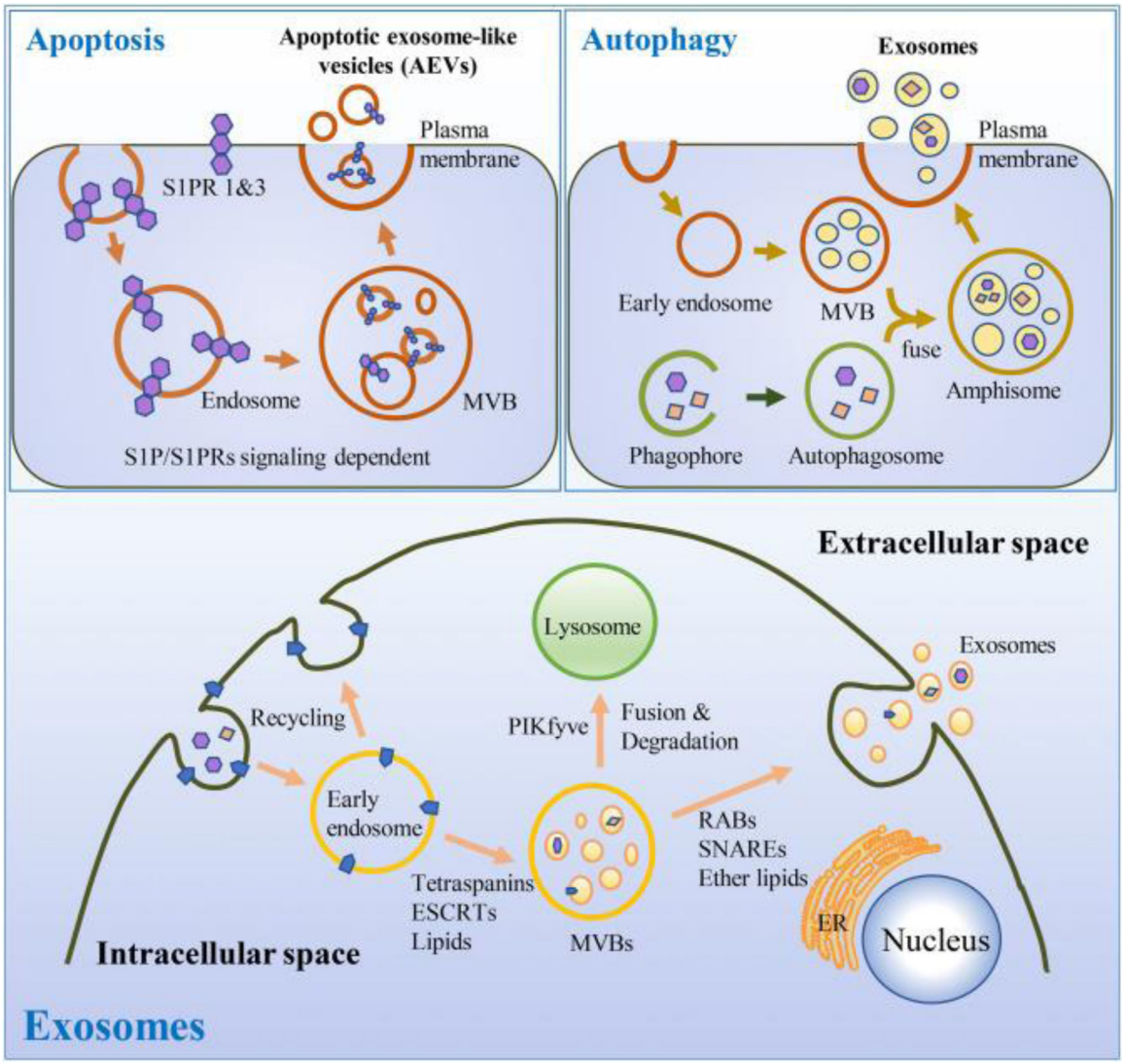

Fig. 1. Schematic illustration of mechanistic generation of exosomes from different cellular events

Brief mechanisms for exosome generation in autophagy [41, 42], and apoptotic [26] cells (upper portion). Molecular and cellular mechanisms that regulate exosome biogenesis and/or release. The process can be divided into three steps: exosome biogenesis, transportation of MVBs to the plasma membrane, and fusion of MVBs with plasma membrane. [161–163] (lower portion).

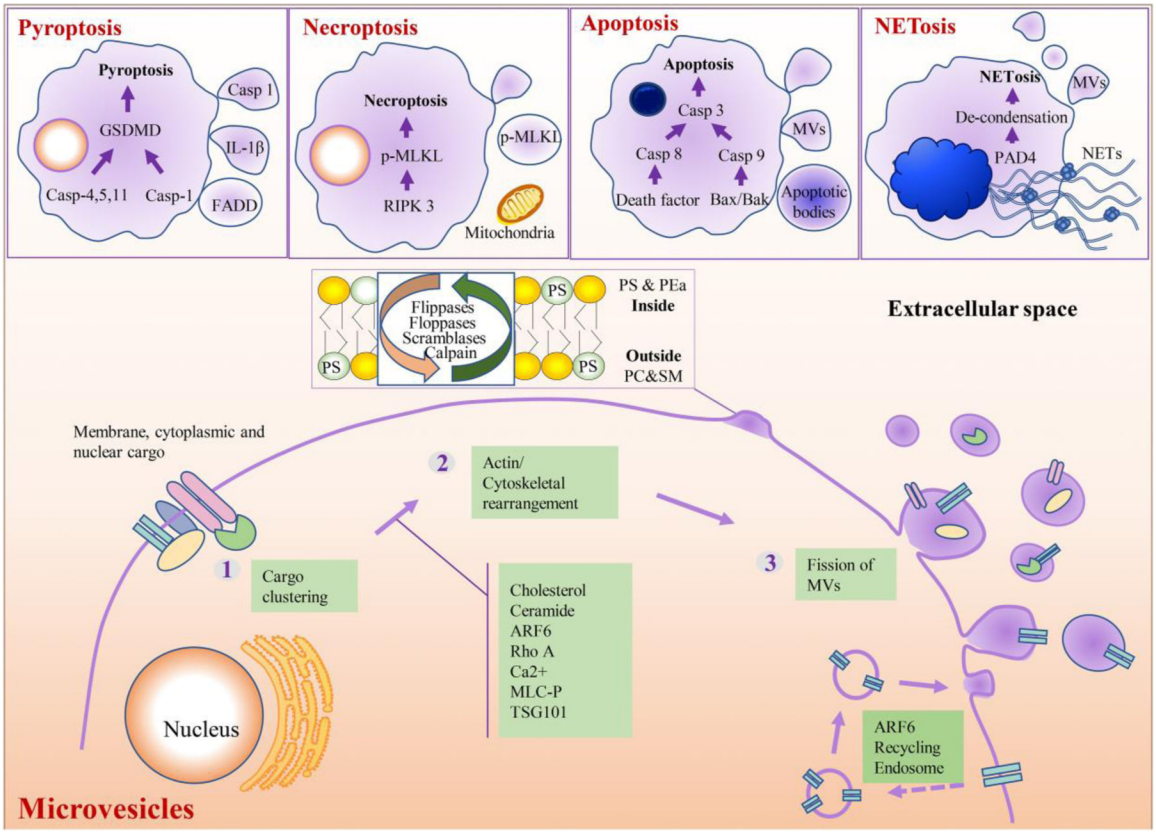

Fig. 2. Schematic illustration of mechanistic generation of MVs from different cellular events

Brief mechanisms of MV generation from pyroptotic [27, 29, 34], necroptotic [30, 38], apoptotic [25], and NETotic [39, 164] cells (upper portion). Molecular and cellular mechanisms that regulate MVs biogenesis and/or release. The process can be divided into three steps. First, membrane-, cytoplasmic- and nuclear- associated cargoes are clustered in specific membrane microdomains of the plasma membrane. Second, the clustered cargoes together with additional machineries promote actin/cytoskeletal rearrangement, phosphatidylserine (PS) exposure, and membrane budding. Then a fission process at the plasma membrane occur. Besides, ARF6 can mediate the secretion of recycling endosome to form MVs. [165–169] (lower portion).

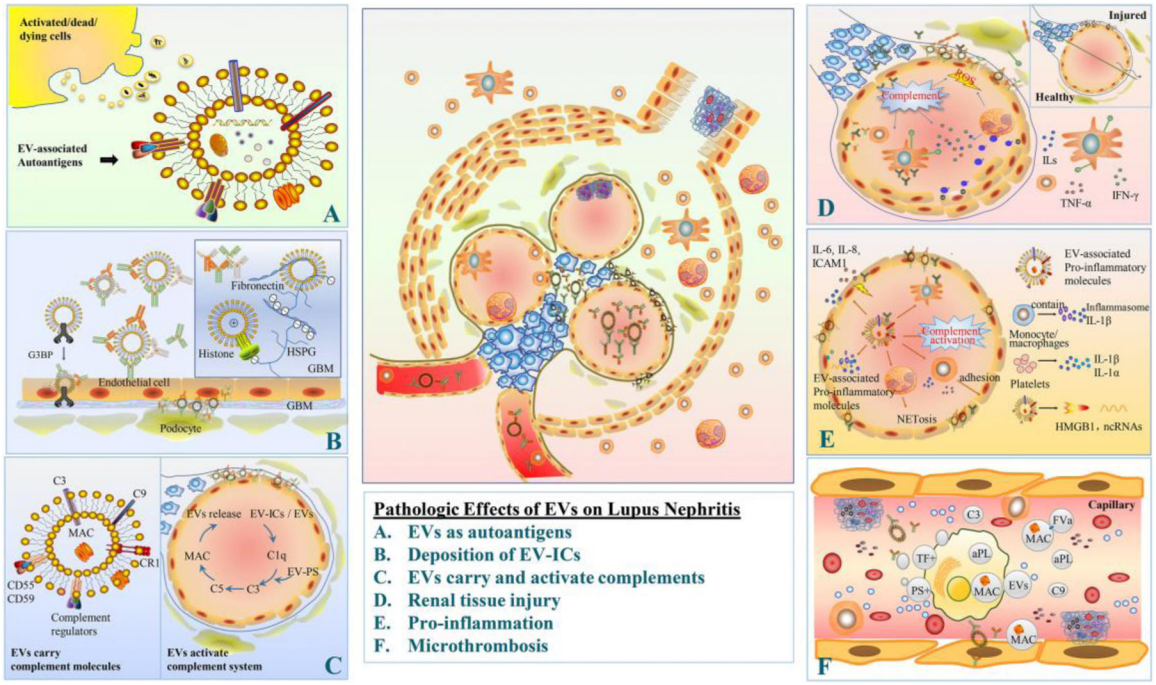

Fig. 3. Potential pathogenic effects of EVs on Lupus Nephritis.

A. EVs: as the source of autoantigens: EVs that are released from activated/dead/dying cells may carry autoantigens, particularly those from nucleus or cytoplasm. **B**. Deposition of EV-ICs: EVs harbor immunoglobulins and complement, thereby forming EVs containing immune complexes (EV-ICs). These EV-ICs along with immune-active structures and molecules deposit in kidney. EVs molecules, including G3BP, histone, fibronectin, may facilitate glomerular IC deposition. **C**. EVs carry and activate complements: EVs may carry many complement molecules, like C3, MAC, complement regulators. On the other hand, EVs may activate complement system through either classical or alternative pathways. Some EVs may activate the classical pathway through binding C1q, while other EVs, i.e. PS containing EVs, may also be activator of complements through the alternative pathway. **D**. Renal tissue damage: EV-associated activated complements and the consequent pro-inflammatory microenvironment causes podocyte injury, foot process effacement, and proliferation of mesangial cells, plus MPO-mediated endothelial damage, therefore leading to proteinuria and glomerular dysfunction. **E**. Pro-inflammation: EVs may carry pro-inflammatory components (cytokines, inflammasome, adhesion molecules, leukotriene, etc.), which further propagate inflammatory responses by activation of other immune cells. In addition, EVs and EV-ICs may activate complement system. **F**. Microthrombosis: PS- and TF-positive EVs as well as the EVs associated with complements are procoagulant, thus contributing to microthrombosis in lupus nephritis.

Table 1.

Summary of the Published Studies about EVs in LN

EVs origin	Cellular origin	Markers	Associated Specific component	Samples	Techniques for analyzing characteristics	Study findings	References	Year
MVs	Podocyte	Annexin V/ Podocalyxin	NA	Urine	Flow cytometry	Higher podocyte-derived MVs in LN and correlate with high activity indices, and increased proteinuria	[151]	2019
MVs	Mainly leukocytes	Platelet CD41a; leukocyte CD45; erythrocyte CD235a	HMGB1/HLA-DR/ CX3CR1	PPP/Urine	Flow cytometry	High frequencies of MP-HMGB1+ in circulation and urine of LN patients. Urinary MP-HMGB1+ could discriminate between patients with active and inactive LN.	[117]	2019
MVs	Mainly platelets	Mitochondria mitoTracker/ TOM20/HK1; Platelet CD42a; T cell CD3	Nucleic acids/IgG	Frozen PPP	Flow cytometry	Patients with signs of ongoing renal lupus activity (BILAG, A-C) had higher levels of mitoMVs and IgG-coated mitoMVs.	[167]	2019
MVs	Apoptotic endothelial cells	KM-2/LG11–2	Acetylated chromatin	PPP	Flow cytometry	MVs containing acetylated chromatin drive ROS independent NET release in SLE patients with active LN.	[168]	2017
MVs	NA	Annexin V/G3BP	G3BP, C1q, immunoglobulins	PPP	Flow cytometry/Co-localization immune electron microscopy(IEM)/ Nano-LC-MS/MS	Co-localization of G3BP positive MVs associated with ICs may deposit in the LN kidneys.	[22]	2015
MVs	Platelet/ endothelial cell	Platelet Annexin V/ CD41; endothelial cell Annexin V/ CD62E	NA	PPP	Flow cytometry	High circulatory platelet microparticles and endothelial microparticles in LN patients can be used as new markers for dysfunctional platelet activation and endothelium.	[169]	2015
Exo	Tubular renal cells	NA	MiR-31/MiR-107/ MiR-135b-5p	Urine	NanoSight/Cryo-TEM/ Western blot	Urinary exosomal miR-135b-5p, miR-107, and miR-31 are promising novel markers for clinical outcomes, regulating LN renal recovery by HIF1A inhibition.	[166]	2020
Exo	NA	CD9/TSG101	Let-7a/MiR-21	Urine	NanoSight/Transmission electron microscopy (TEM)/Western blot	Down-regulation of let-7a and miR-21 in urine exosomes from LN patients during disease flare.	[164]	2018
Exo	NA	CD9/CD81	MiR-3135b/ MiR-654–5p/ MiR-146a-5p	Urine	Transmission electron microscopy (TEM)/ Western blot	LNIV-CC has a unique miRNA expression profile of urinary exosome and miR-3135b, miR-654–5p and miR-146a-5p in urinary exosomes could predict LNIV-CC.	[170]	2018
Exo	NA	CD9/AQP2/TSG101	MiR-29c	Urine	Electron microscopy Western blot	Decreased miR-29c in exosomes in LN is associated with increased renal chronicity and predict early renal fibrosis in LN.	[165]	2015
Exo	NA	CD9/TSG101	MiR-146a	Urine	Transmission electron microscopy (TEM)/ Western blot	Exosomal miR-146a discriminates the presence of active LN.	[162]	2015

EVs origin	Cellular origin	Markers	Associated Specific component	Samples	Techniques for analyzing characteristics	Study findings	References	Year
Exo	NA	NA	MiR-26a	Urine	NA	Increased miR-26a levels in urinary exosomes may serve as a marker of injured podocytes in LN.	[163]	2014
ABs	NA	NA	NA	Renal tissue	Periodic acid-Schiff methenamine silver	The presence of large numbers of apoptotic bodies in the glomeruli of Clq-deficient mice.	[171]	1998
ABs	NA	NA	NA	Renal tissue	Electron microscopy	The presence of apoptotic cells and apoptotic bodies in proliferated mesangial areas and within the glomerular capillaries.	[172]	1995

MVs (Microvesicles), mitoMVs (mitochondria MVs), Exo (Exosomes), ABs (Apoptotic Bodies), PPP (platelet poor plasma), LNIV-CC (Type IV lupus nephritis-cellular crescent), ROS (Reactive oxygen species), LN (Lupus Nephritis), ICs (Immune Complexes), HMGB1 (High-mobility group box 1), G3BP (Galectin-3 binding protein), HK1 (anti-hexokinase 1).