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# Role of Toll-like receptors in exosome biogenesis and angiogenesis capacity

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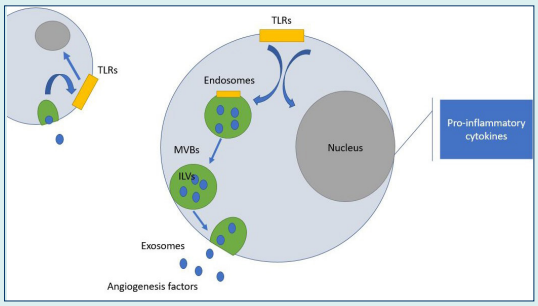
**Abstract**




Adaptive inflammation consists of multiple cellular changes and molecular reactions to protect host cells against several pathological conditions. Along with the activation of varied immune cells, the production and secretion of cytokines arrays can regulate the progression of inflammatory response in a paracrine manner. Among different molecular cascades, Toll-like receptors (TLRs) are activated in response to several pathological conditions and damage signals. It has been indicated that extracellular vesicles, especially exosomes (Exos) are key bioshuttles with specific cargoes and are involved in cell-to-cell communication. The role of Exos in the initiation, progression, and cession of inflammation has been previously addressed in terms of cytokine transmission. Whether and how the activation of TLRs can alter the Exo biogenesis and angiogenesis potential in immune cells and endothelial cells (ECs) remains to be elucidated. Here, the cross-talk between the TLRs, Exo biogenesis, and angiogenesis has been highlighted.

## Introduction

Among different signaling cascades, Toll-like receptors) TLRs (and relevant downstream effectors participate in several cell bioactivities. These receptors belong to the pattern recognition receptors family (PRRs).[1] In response to exogenous pathogens and damage signals, the activation of PRRs triggers a series of molecular pathways leading to the production of pro-inflammatory cytokines.[2] Of 13 mammalian types of TLRs, murine and human cells can express TLR1-10, 12, and 13.[3] Notably, the function of all TLRs was defined previously except the TLR10 (Fig. 1). Based on subcellular location, TLRs are classified into two groups. The cell plasma membrane-bound TLRs such as TLR 1, 2, 4, 5, 6, and 11 have the potential to bind microbial compounds, while endosomal TLRs such as TLR 3, 7, 8, and 9 can attach to intracellular pathogens.[4] From a structural viewpoint, TLRs possess three distinct domains. The N-terminal extracellular domain is composed of a hydrophobic leucine-rich repeat, namely LRR, with the potential to recognize several pathogens. The middle part possesses a single helical motif that passes through a plasma membrane, and finally, the conserved cytoplasmic domain is juxtaposed to the Toll/interleukin-1 receptor (TIR) complex.[5]

TLR signaling is stimulated after direct interaction of TLRs with TIR-domain containing adaptor protein, namely myeloid differential factor 88 (MyD88) (Fig. 1).[6] It is believed that the attachment of specific ligands such as pathogen-associated molecular patterns (PAMP) such as RNA viruses, several bacteria species, and damage-associated molecular patterns (DAMPS) like miRNAs, heat shock proteins (HSPs), and high mobility group box 1 (HMGB1) can promote TLR signaling pathway.[7] Bacterial lipopolysaccharides (LPS) can stimulate the TLR

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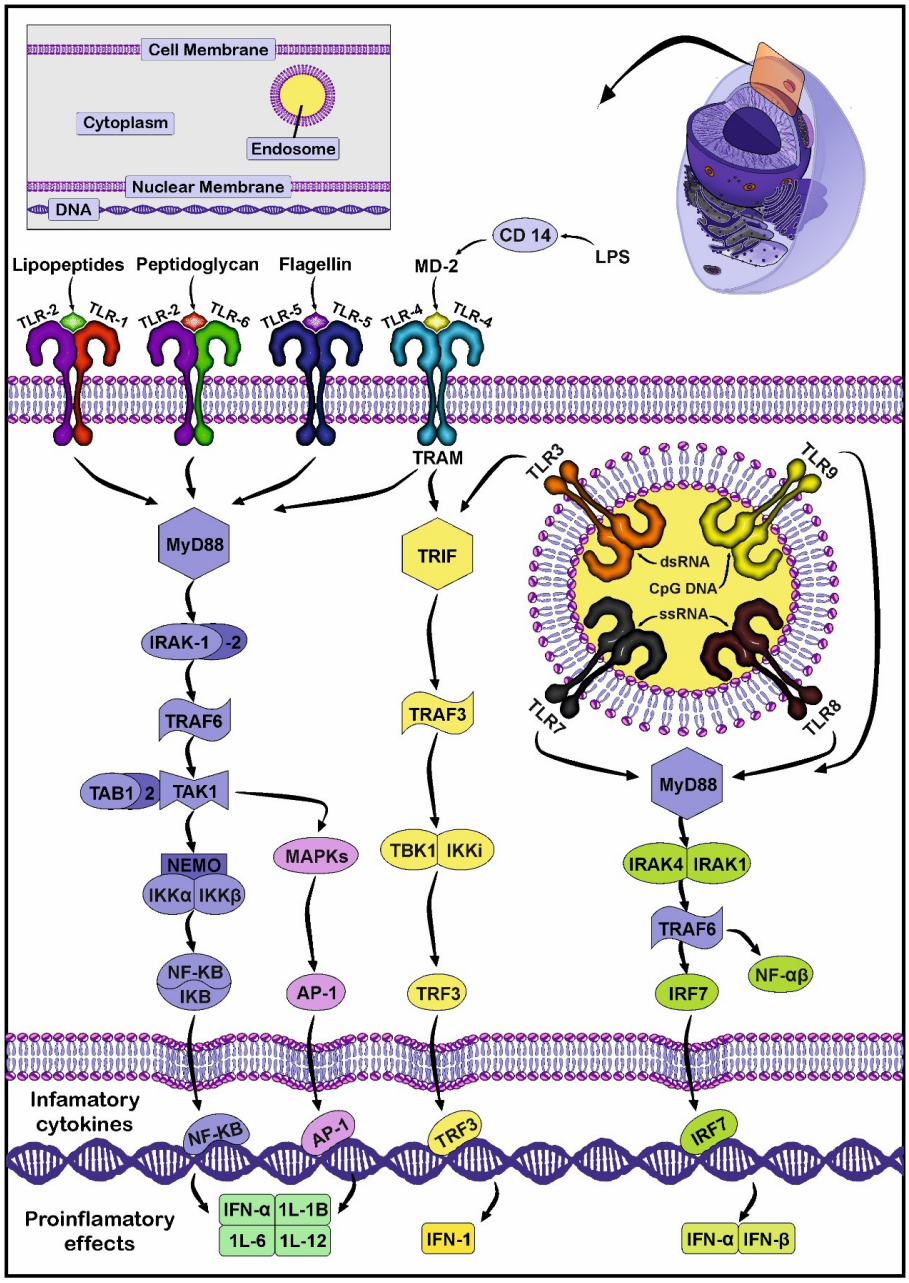


**Fig. 1**. TLR signaling pathway. The homodimers of TLR5, TLR4, and heterodimers TLR2-TLR1 or TLR2-TLR6 recognize DAMPs and PAMPs on the surface of the plasma membrane. Inside the cells, TLR3, TLR7-TLR8, and TLR9 are located on the membrane of the endosomes and identify various intracellular genetic components. Following the close interaction of ligands and TLRs, downstream molecular cascades are initiated in MyD88-dependent or MyD88-independent (TIRAP axis) manners. In the MyD88-dependent signaling pathway, the recruitment of effectors such as IRAK4 and IRAK1/2 leads to the engagement and activation of TRAF6 and TAK1 complex, respectively. In the MyD88-dependent pathway, factors belonging to the IRAKs family such as IRAK4, and IRAK1/2 are recruited and phosphorylated. In the next step, IRAK1 can interact with TRAF6 which is followed by the addition of TAK1. TAKI can stimulate MAPK, and facilitate the translocation of NF-kB into the nucleus, leading to the production of several pro-inflammatory cytokines. In an alternative pathway, TLR3 and 4 can exclusively bind to TRIF without the participation of MyD88, resulting in the entry of TRF3 into the nucleus and INF expression. Created by CorelDRAW Graphics Suite Ver. 250.0.0.230.

co-receptor CD14 and thus downstream effectors.[8] Results have shown that the release of endogenous molecules after tissue injury can be easily recognized by different TLR types. Among them, HSP60 can activate TLRs at the early stages of tissue injury and inflammation.[9] Based on the recruitment of distinct adaptor proteins, the TLR signaling pathway is classified into myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent axes. The latter pathway is promoted by TIR-domain-containing adaptor-inducing interferon-β (TRIF).[10] Previous molecular investigations have shown that only TLR 3 can bind exclusively to TRIF without the activation of MyD88. By contrast, TLR4 can engage both MyD88 and TRIF effectors while other TLR types are dependent on MyD88 activity.[11] Upon activation of the TLR signaling pathway, the transcription of certain factors such as nuclear factor-

κB (NF-κB) mitogen-activated protein kinase (MAPK), and pro-inflammatory cytokines are stimulated (Fig. 1). Of note, TLRs in hetero or homo-dimer structures can bind to the target molecules.[12] The close interaction of PAMPs or DAMPs with TLRs activates the TLR/MyD88 axis. In the next step, MyD88 recruits IL-1R-related kinases (IRAKs) family such as IRAK4 and 1. Both IRAK4 and 1 are phosphorylated, followed by the separation of IRAK1 from MyD88 and close interaction with TNF receptor-associated factor 6 (TRAF6).[13,14] Thereafter, transforming growth factor-β-activated kinase 1 (TAKI) and subunits TAK1-binding protein (TAB1, 2, and 3) attach to TRAF6.[15] The activated TAK1 can promote two signaling cascades, such as phosphorylation of IκB kinase (IKK) and activation of mitogen-activated protein kinase (MAPK).[16] Under normal circumstances, IκB can inhibit nuclear factor NF-κB. After the activation of the IKK complex, IκB is phosphorylated and subsequently degraded. In the latter step, NF-κB translocates into the nucleus to up-regulate the expression of pro-inflammatory cytokines.[17] In an alternative pathway, the activation of the MAPK signaling pathway triggers activator protein 1 (AP1). This factor in collaboration with NF-κB increases the synthesis of IL-1, 6, and TNF-α.[18] The activation of the TRIF pathway by TLR3 and 4 stimulates another signaling pathway[16,19] It should be noted that TRIF and TRAM (TRIF-related adaptor molecule) can be activated via the MyD88-independent pathway. TRAM acts as an adapter protein and connects TLR to TRIF, leading to TAK1, TRAF6, and NF-ƙB activation.[18] It was suggested that the activation of TRAF6 leads to the promotion of TBK1 and IKK-ε to phosphorylate interferon regulatory factor 3 (IRF3). The procedure is followed by the translocation of phosphorylated IRF3 into the nucleus and the expression of type 1 interferon.[18,20]

## Exosome biogenesis

Exosomes (Exos) are nano-sized extracellular vesicles with the potential to carry biological cargo such as lipids, proteins, short-length DNAs, and various RNA types from the host cells to acceptor cells. Based on different studies, Exos with a mean diameter size of 30-150 nm, are originated from the endosomal system.[21-23] The phenomenon of Exo biogenesis is a multi-step process in which several intraluminal vesicles (ILVs) are generated via the invagination of plasma membrane inside late endosomes and multivesicular bodies (MVBs) (Fig. 2).[24-26] In the next steps, the fusion of MVBs with the plasma membrane leads to the release of ILVs into the extracellular environment, hereafter known as Exos.[27] Two distinct pathways have been proposed for MVBs in the latter steps. First, MVBs can fuse with lysosomes and are subjected to proteolytic degradation.[28] Besides, MVBs can be oriented to the cell membrane where direct physical contact and fusion lead to the release of ILVs into the extracellular matrix (ECM). Evidence revealed that various factors, such as the endosomal sorting complex required for transport (ESCRT), lipids (ceramides), and tetraspanins participate in ILV formation within MVBs.[29] Cargo sorting is orchestrated via ESCRT-dependent or ESCRT-independent pathways.[26] ESCRT is a cytoplasmic protein that generates coated subdomains on the surface of endosomes. These features facilitate membrane budding and the formation of ILVs. From the molecular structure, the ESCRT complex is composed of four certain subsets, including ESCRT-0, -I, -II, and -III, that are in close relation with specific proteins ALIX, TSG101, and VPS4.[30] These factors can help in the sequestration of certain cargo into the lumen of ILVs.[30]

Generally, the ESCRT machinery has the main role in attaching, sorting, and clustering ubiquitinylated proteins into the MVBs.[31] To initiate these procedures, ESCRT-0 recognizes and sequestrates ubiquitinated proteins into the endosomal membrane. In the next steps, the activation of ESCRT-I and -II subsets predisposes membrane deformation and invagination of ILVs.[31,32] The close interaction of Alix with this complex promotes the recruitment of ESCRT-III. The addition of ESCRT-III accelerates the release of *de novo* ILVs into the endosomal lumen. The collaboration of Alix with exosomal proteins like the ESCRT complex can play a key role in budding and cutting the endosomal membrane through interaction with syndecan.[33] Along with these changes, energy supply via accessory proteins such as Vps4 causes the recycling of ESCRT machinery.[34] In addition to the ESCRT-dependent pathway, components of the ESCRT-independent pathway such as ceramides can also help the ILV generation. For example, the enzymatic activity of sphingomyelinase leads to the production of ceramide from sphingomyelin, and accumulated ceramide levels contribute to the formation of ILVs within MVBs.[35,36] Other components of ESCRT-independent pathways are tetraspanins, which are involved in ILV formation. Tetraspanins consist of a large number of protein complexes in the plasma membrane of Exos, lysosomes, and endosomes.[37,38] It has been thought that Exos harbor large contents of tetraspanins with several transmembrane domains.[39] Tetraspanins are in close association with each other and different signaling proteins.[40] Several studies have applied tetraspanins as integral biomarkers for Exo characterization and immunophenotyping. Besides, these factors are involved in migration, signaling, fusion, intercellular adhesion, and other functions.[29,41] Tetraspanins mainly CD9, CD37, CD53, CD63, and CD81 can foster the generation of ILVs using various mechanisms. To be specific, CD81 facilitates Rac GTPase function while CD9 promotes the fusion process with the plasma membrane and has an important role in the release of exosomal β-catenin.[42] Notably, CD63 is abundantly present in the exosomal membrane and actively participates in the biogenesis of Exos.[43] The

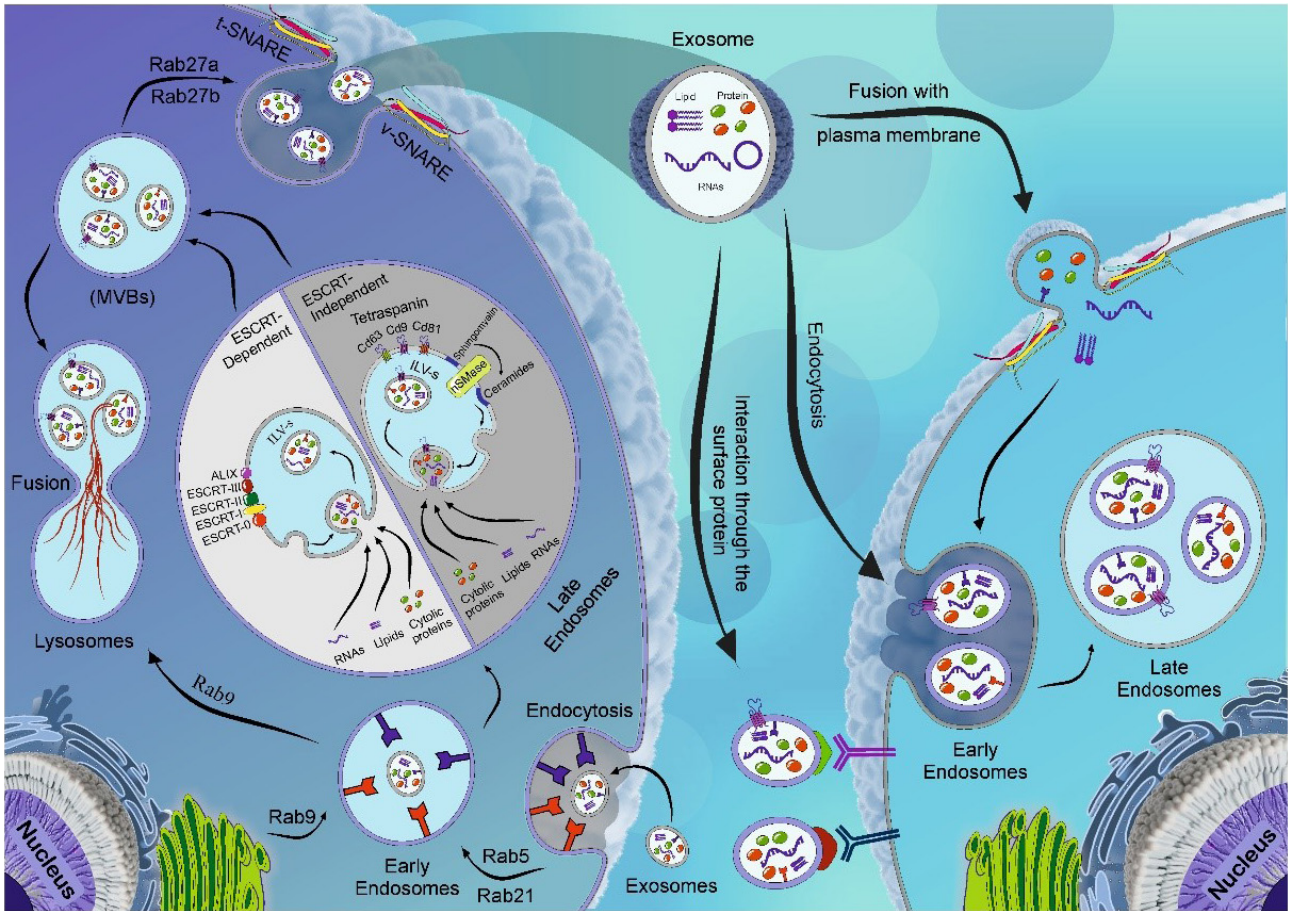


**Fig. 2.** Schematic illustration of Exo biogenesis and abscission. Different effectors participate in the biogenesis of Exos. Following the internalization of cargo into the target cell, the early endosomes are generated. Early endosomes can mature into late endosomes after packing several signaling molecules such as proteins, lipids, and nucleic acids. This activity is performed via the invagination of endosomal membranes, resulting in the formation of numerous intraluminal vesicles (ILVs). In the next step, later endosomes mature into MVBs. The process of cargo sorting into the ILVs is done via the participation of ESCRT-dependent or ESCRT-independent pathways. MVBs can fuse with the lysosomes and undergo enzymatic digestion or maintain physical contact with the plasma membrane to release ILVs into the ECM, where hereafter known as Exos. Effectors such as RABs, v-SNARE, and t-SNARE are required for the fusion of MVBs with the plasma membrane. The released Exos can be guided to close or remote cells through direct fusion, receptor-mediated fusion, and endocytosis. Created using CorelDRAW Graphics Suite Ver. 250.0.0.230.

existence of distinct tetraspanin membranes such as CD55 and CD59 protects Exos from the complement system.[44,45] Recent studies state that the ESCRT-independent pathway can also modify exosomal cargo.[46] The secretion of Exos from the host cells is associated with the activity of several factors like molecular switch Rab GTPase, membrane fusion complexes such as soluble NSF attachment protein receptors (SNAREs), cytoskeleton proteins (actin, and myosin, and motor proteins kinesin, and dynein).[47,48] As mentioned earlier, the Rab is a subset of the RAS family with GTPase activity. This protein is located on the cytosolic side of the plasma membrane and plays a crucial role in regulating molecular pathways and Exo trafficking.[49] The stimulation of both RAB and SNARE complexes expedites the MVB docking and fusion with the plasma membrane, leading to Exo secretion (Fig. 2).[50] For instance, Rab5 regulates the release of Exos by forming and fusion early endosomes. Rab27 regulates the movement and attachment of secretory vesicles with the plasma membrane via physical contact with specific factors.[51] Likewise, microtubules and molecular motors are important in transporting MVBs towards the plasma membrane.[52] These factors are required to transport, dock, and assimilate MVBs with the plasma membrane.[53] A zipper-like structure is formed by pairing SNAREs (v-SNARE and t-SNARE) can promote the abscission phenomenon and the release of Exos ECM.[25]

## TLRs and exocytosis

As a common belief, the induction of the TLR signaling pathway causes the translocation of NF-κB into the nucleus with the involvement of several factors and enzymes. It is thought that NF-κB activation can be done through proteasome-mediated proteolysis of ubiquitin (Fig. 3).[54,55] Ubiquitin has a critical role in pro-inflammatory responses after cell exposure to microbial products and an array of cytokines. Under physiological conditions, NF-κB is inhibited by IκB within the cytosol. Soon after the attachment of stimulatory ligands to TLRs, IRAK1, and 2 are phosphorylated to stimulate TRAF6.[56]

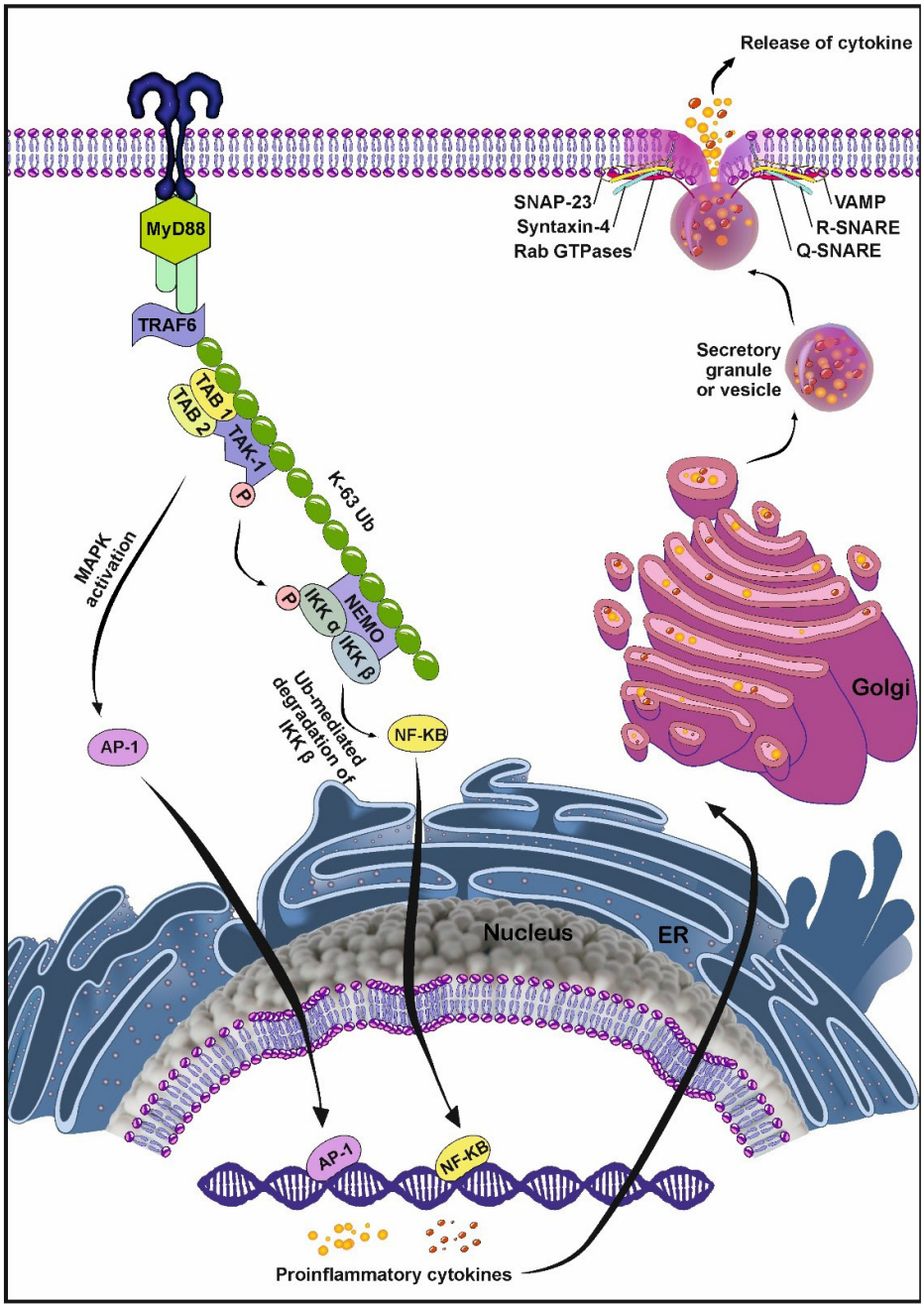


**Fig. 3.** Polyubiquitination systems can connect TLRs with exocytosis. After ligand-TLR interaction, TRAF6 is activated via association between IRAK4/1 and TRAF6 in a MyD88-dependent manner. TRAF6 functions as a ubiquitin ligase and stimulates the synthesis of K63-linked polyubiquitin. The physical attachment of TRAF6 to the ubiquitin lysine residue activates protein kinase TAK1. TAK1 activates IκB kinases (IKKα, IKKβ, IKKγ) and phosphorylates IκB-α. Phosphorylation and ubiquitination of the protein inhibitor IκB lead to its degradation. Afterward, NF-κB is activated by the degradation of IκB through the ubiquitination pathway with the translocation of NF-κB into the nucleus. NF-κB in the nucleus promotes the synthesis of numerous pro-inflammatory cytokines. In the next step, pro-inflammatory cytokines synthesized in the ER and Golgi complex, are transported to the cell surface. A set of SNAREs is required for the binding of secretory granules to the plasma membrane and the release of cytokines from the granules. An R-SNARE in the membrane of the secretory organelle associates with a Q-SNARE-like complex (SNAP-23 and syntaxin-4) in the target membrane, forming a 4-helix coil structure which is necessary for the release of cytokines. Created by CorelDRAW Graphics Suite Ver. 250.0.0.230.

In the following steps, the binding of TRAF6 to ubiquitin lysine residue *per se* promotes the kinase activity of TAK1. TAk1 and I kappa B kinase (IKK) simultaneously bind to non-canonical protein ubiquitination k-63 via phosphorylation and ubiquitination of the IKKg subunit (NEMO). These features lead to proteasomal degradation of IKB.[57-59]

Several studies have shown that secretory nanovesicles are cytokine-transporting vesicles.[60] Upon the production of secretory cytokines in the endoplasmic reticulum (ER), they are transferred to the Golgi apparatus and packed. After that, cytokines are released from the cell by secretory vesicles via exocytosis.[61,62] In this pathway, small GTPases belonging to the Rab family are involved

in the formation, movement, attachment, and fusion of vesicles to target membranes.[63] Some studies indicate that secretory granules are physically connected to the plasma membrane in response to TLR stimulation via the activation of SNAREs as intracellular proteins.[61,64]

SNAREs are a family of proteins with conserved structures and form a complex in combination with membrane proteins that fuse docked bilayer lipids.[65] Depending on the type of amino acid in the central core, SNAREs fall into two subfamilies: Q-SNARE with glutamine (Q) residue and R-SNARE with arginine (R) residue as a central functional residue in motif SNAREs.[66] A Q-SNARE complex contains syntaxin, synaptosomal-associated protein (SNAP), and R-SNARE contains VAMP. To form four helical bundles for membrane fusion, one R-SNARE on the secretory vesicle and two or three Q-SNAREs on the plasma membrane are essential.[67,68] Q- and R-SNAREs pairing is important in trafficking cytokines from the host cells.[69] By interacting subfamily of SNAREs from the N-terminus to the C-terminus of the opposite membrane, the α-helix bundle (zipper) is formed, which is called trans-SNARE. The Zipper complex provides the energy required to keep hydrophobic lipids in the plasma membrane and overcome hydrostatic pressure.[70]

## Effect of TLRs on mitochondria in myocardial infraction

During myocardial infarction, DAMPs are released with the death of cardiac cells. DAMPs stimulate the resident immune cells by binding to cardiomyocyte pattern recognition receptors (PRRs).[71] Recruitment of immune cells followed by the release of inflammatory cytokines leads to inflammation in the infarcted area.[72] The heart as an energy-demanding organ has a large number of mitochondria in each cardiomyocyte. Necrotic cardiomyocytes release mitochondrial DAMPs (mtDAMPs) into the cytosol or extracellular space.[73] Studies have shown that mtDAMPs can induce inflammatory factors through multiple signaling pathways, including Toll-like receptor (TLR) 9, cGAS- (cyclic guanosine monophosphate-adenosine monophosphate synthase-) STING, and pyrin domain-containing protein 3 (NLRP3). Released mtDAMPs are oxidized when exposed to reactive oxygen species (ROS) and oxidized mtDAMPs can trigger TLRs.[74] During prolonged ischemic and hypoxic conditions, large amounts of free radicals are produced, and high amounts of cardiomyocyte mtDNA are recognized by PRRs.[75,76] Research shows that endogenous DAMPs released from damaged cardiomyocytes bind to TLR receptors in intact cardiomyocytes. TLR-9 has been identified as the most important mtDNA receptor through the identification of an unmethylated CPG motif in mtDNA structure.[77] The activation of endosomal TLR-9 by mtDAMPs stimulates NF-κB in the MyD88-dependent pathway. As the main regulator of inflammation, NF-κB plays a role in the increased expression of pro-inflammatory cytokines, tissue remodeling, and immune responses.[76] In addition, the elevation of ROS after mitochondrial injury causes oxidative stress and the secretion of chemokines and pro-inflammatory cytokines (IL-6, and -8).[78] Besides, the increase of mtDNA and mitochondrial ROS cardiomyocyte cytoplasm contributes to the formation of NLRP3 inflammasomes. The inflammasomes are the main source of interleukin 1β (IL-1β) and (IL-18) and play an important role in the recruitment of inflammatory cells and the promotion of cardiomyocyte death.[81,82] In this pathway, caspase-1 regulates the inflammatory factors such as IL-1β and IL-18. Activation of signaling pathways by mtDAMPs causes more damage to mitochondria and thus more mtDAMPs are released.[83] Another inflammatory pathway activated by mtDNA is cyclic GMP-AMP (cGAMP) synthase-(cGAS-) STING pathway (cGAS-STING). The mtDNA released in the cytoplasm recognizes the cGAS-STING pathway, after that it leads to STING homodimerization and activation. Further phosphorylation of IκB activates the transcription factor NF-κB.[83, 84] Previous data have shown that circulating mtDNA plays a significant role in the development of heart diseases by activating inflammatory pathways.[85]

## Impact of Inflammation on exosome production and release

The findings are consistent with the fact that several inflammatory stimuli such as metabolic disease and pathological conditions can affect the Exo biogenesis and secretion.[86,87] On the other hand, exosomal cargo can regulate the extent and duration of inflammatory response under physiological and pathological conditions.[88] In some inflammatory diseases, Exos induce pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α through PAMP receptors such as TLRs.[89] For example, IL-1β cytokine secretion is responsible for fever and leukocyte recruitment after the initiation of inflammation.[90] In response to intracellular damage and endogenous pathogens, the formation of protein complexes namely NLRP3 inflammasomes is initiated via canonical and non-canonical pathways.[91] In the canonical axis, the activation of IL-1β, Caspase 1, and IL-18 are the main fundamental factors that lead to the inflammasome-associated inflammatory response.[92] Recent works have indicated the importance of Caspase 1 activation on intracellular trafficking of vesicles via the regulation of Rab-interacting lysosomal protein (RILP).[93] In an experiment conducted by Wozniak and colleagues, THP-1 macrophages priming with bacterial LPS (inflammasome inducer) and ATP (inflammasome activator) can increase RILP cleavage, leading to the activation of Exo cargo sorting and secretion.[93] Based on the data, these effects were blunted when the non-cleavable form of RILP was expressed in the host cells.[93]

The activation of RILP is an appropriate strategy to close the MVBs' direction toward the cell membrane. Along with these changes, fragile X mental retardation protein (FMRP) with KH domain can sort miRNAs with AAUGC motif onto the ILVs inside the MVBs via the collaboration with ESCRT complex after Caspase 1 cleavage.[93] Of note, circulating Exos harbors high contents of FMRP and IL-1β in patients with non-alcoholic steatohepatitis compared to healthy counterparts.[93] To support the existence of a direct relationship between Exo secretion and inflammation, Chen et al found that pre-treatment of mouse RAW264.7 macrophages with dexamethasone and incubation with bacterial LPS reduced Exo release compared to the control LPS group. These data coincided with the reduction of exosomal inflammatory miRNA-155.[94] Under metabolic diseases, the stimulation of the endosomal system and dysregulation of lysosomal activity can affect the host cell's capacity to release the Exos.[95] In this regard, Huang and co-workers examined the effect of homocysteine on Exo biogenesis and the secretion of podocytes in a mouse model.[95] They claimed that in response to hyperhomocysteinemia, NLRP3 inflammasomes are activated and sequestrated onto the ILVs in later endosomes and MVBs. It was suggested that simultaneous activation of lysosomal acid sphingomyelinase increases the intracellular levels of sphingolipids and ceramides, resulting in the reduction of MVB-lysosome interaction and an increase of Exo release from podocytes. The inhibition of acidic and neutral sphingomyelinase activity using Amitriptyline and GW4869 can stimulates MVB-lysosome interaction and Exo biogenesis, respectively.[95] Simultaneous treatment with mTORC1 inhibitor, rapamycin, triggers the fusion of MVBs with lysosomes and cargo degradation.[95] Data indicated that regulated inhibition of inflammatory Exo secretion can alleviate glomerular inflammation under pathological conditions. In line with these changes, the inflammatory response can expedite the process of Exo biogenesis and release into the ECM space. Due to changes in cargo sorting into Exos and secretion of certain inflammatory factors, the induction of intracellular lysosomal degradation is touted as an effective approach to the expansion of inflammation and pathological changes to juxtaposed sites. Likewise, Ye and co-workers indicated that the induction of albuminuric nephropathy in mice using Adriamycin led to enhanced Exo secretion by Rab27a activity.[96] The prolonged exposure of tubular epithelial cells to albumin increased the transcription of interferon regulatory factor 1, a factor that regulates the expression of GTPase Rab27a.[97] Based on the data, Rab27a silencing increased lysosomal degradation of Exo and intracellular albumin and diminished the production of cellular inflammatory cytokines.[96] It is postulated that phagocytes and inflammatory cells can exert their effective role during pathological conditions via the regulation of Exo biogenesis. In support of this notion, systemic levels of TNF-α, IL-1β, IL-6, and cardiovascular pathologies were significantly reduced after injection of endotoxin in mice pre-treated with Exo biogenesis blocker GW4869.[98] It should not be forgotten that the release of Exos can also occur in response to the intracellular accumulation of noxious and undegraded materials to prevent cell atresia.[99] Guix and co-workers declared that the progressed aging process in neurons can distort the normal activity of Exo biogenesis and release.[100] By the progression of aging changes, the number of large-sized MVBs, and lysosome-associated multilamellar bodies increased along with the lysosome heterogeneity.[100] Also, the number of Exos with small diameter sizes increased in cell culture supernatant, reflecting the enlargement of MVBs inside the cytosol. These effects are associated with reduction of cholesterol-transporter NPC1 protein, resulting in the accumulation of cholesterol and GM2 in endosomal vesicles and lysosomes. Under such conditions, the reduced activity of Akt-mTOR axis and suppression of autophagosomes with lysosomes are evident inside the affected cells.[100] Of note, Exos can also from in dying apoptotic cells and cells exposed to severe pathological conditions.[101] Immunofluorescence staining indicated the formation of $CD63^+$microvesicle in apoptotic HeLa cells induced by staurosporine in a caspase-dependent manner. Based on data obtained from a study conducted by Park and colleagues, the activated sphingosine-1-phosphate/ sphingosine-1-phopshate receptors are involved in the production and release of ILVs in apoptotic HeLa cells.[101]

Whether the contact of immune cells with normal and/or cancer Exos can lead to different inflammatory responses is at the center of the subject. It was suggested that palmitoylated proteins on breast cancer cell Exos can stimulate NF-κB and increase the release of pro-inflammatory cytokines such as TNF-α, IL-6, chemokine ligand 2 (CCL2), granulocyte colony-stimulating factor (GCSF).[102,103] In this regard, the entry of cancer Exos to leukemic monocytes (THP-1 cells) increases the TNF-α. The attachment of this factor to TLRs (2 and 4) promotes NF-κB and STAT3. It has been well-known that the reciprocal interaction of NF-κB and STAT3 regulates the inflammatory response during neoplastic changes.[104,105] These features unveil the potent paracrine activity of Exos between the tumor cells and tumor-associated macrophages (TAMs). Exos released by breast and gastric tumors triggers the phenotype acquisition of macrophages toward M1.[102] Certain molecular signatures such as Annexin A2 in the exosomal membrane directly activate the p38MAPK/NFκB/STAT-3 axis and production of IL-6 and TNF-α.[106] In addition to the regulatory effect of Exo on the inflammatory response, the attachment of LPS or angiotensin II can force cardiac macrophages to stimulate inflammatory status in fibroblasts via miR-155-bearing Exos.[107] Other genetic materials such as exosomal

circ_0075932 increase Aurora A kinase in dermal keratinocytes after binding to PUM2 protein, resulting in inflammatory responses. Under these conditions, the secretion of MMP-2, and -9 is induced following the activation of the NF-κB signaling pathway. It is thought that these factors can increase tumor cell resistance to therapeutic agents.[108] The release of EVs from hypoxic endothelial cells (ECs) and cardiomyocytes led to *in situ* accumulation of IL-6 and chemokines CCK2 and CCK7.[109] Unexpectedly, the induction of inflammation via TLRs in the tumor niche can lead to the inhibition of tumoricidal effects. For instance, exosomal miR21 and mir290 from cancer cells increase the migration of cancer cells after binding to TLR-7 and -8.[110] Within the pulmonary niche, EC Exos intensify inflammation in COVID-19, COPD, and asthma subjects via local accumulation of IL-6 (Fig. 4).[111,112]

## TLR and angiogenesis potential of ECs

Angiogenesis is a vital biological process in multicellular creatures under physiological and pathological conditions.[113] The term angiogenesis points to the formation of new blood vessels from the preexisting vascular networks in response to hypoxia and ischemic conditions.[114] Compared to angiogenesis, the term vascularization refers to the generation of *de novo* blood vessels with the participation of endothelial progenitor cells (EPCs).[115] It is thought that the angiogenic switch coincides with the activation of ECs and the formation of neovessel sprouts toward cytokine gradients.[116] Whether and how the TLR signaling pathway can regulate the activity of ECs under pathological conditions is at the center of debate. The role of TLR in terms of angiogenesis can be studied in two different aspects; the modulation of TLRs in ECs, and ECs response to non-ECs after

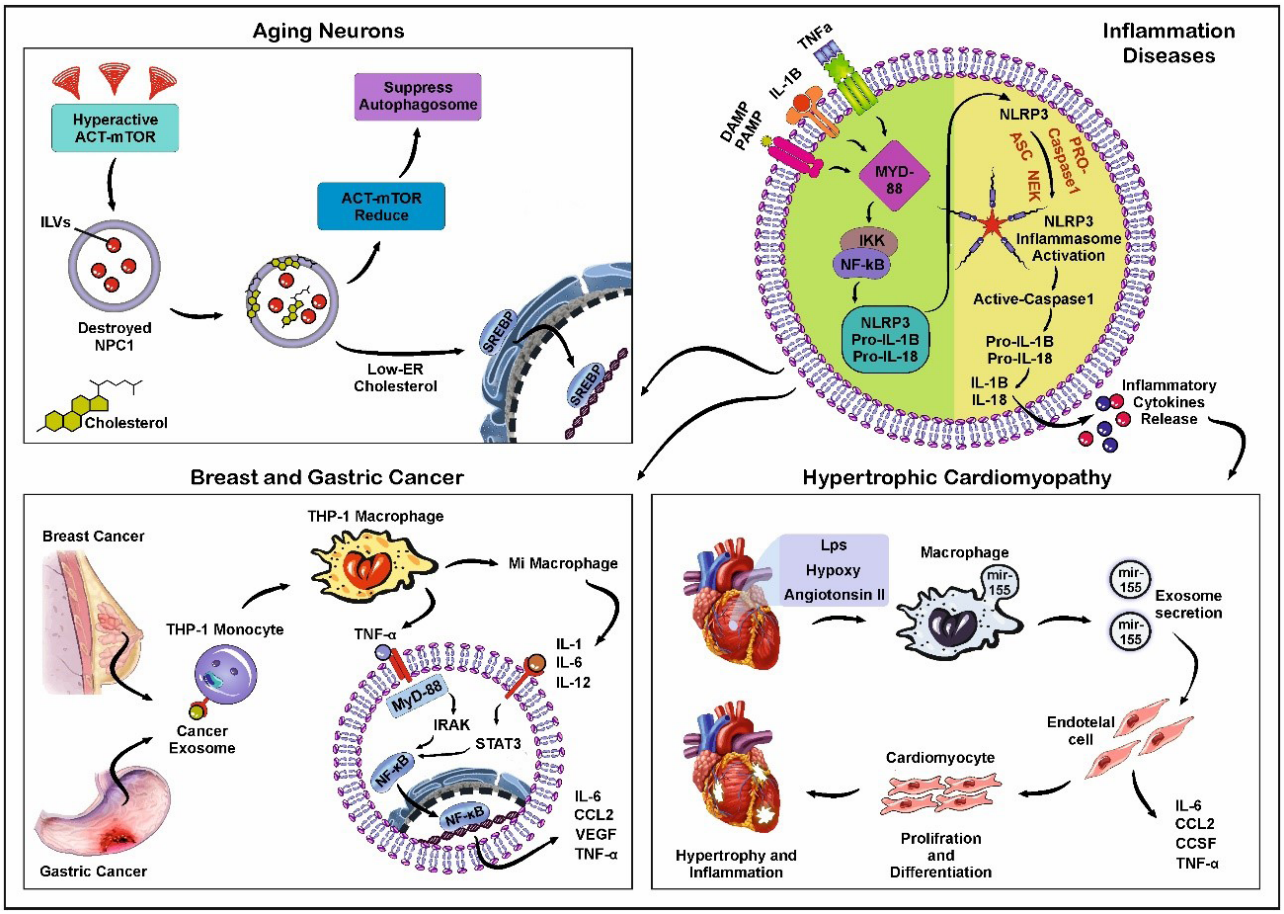


**Fig. 4.** Schematic illustration of inflammatory diseases. The activation of NLRP3 Inflammasomes (Upper right). The binding of DAMPs, PAMPs, IL-1B, and TNF-α to cognate cell surface receptors leads to the up-regulation of NLRP3, pro-IL-1, and pro-IL-18 through the activation of NF-κB signaling axis. Some factors can activate NLRP3 inflammasomes independently. After activation of the NLRP3 inflammasomes, Caspase-1 can process pro-IL-1 and pro-IL-18 into their mature forms. Scheme of the alterations found in aging neurons (Upper left). Hyperactivation of the AKT/mTOR pathway during neuronal aging leads to the degeneration of type C protein (NPC1) and accumulation of cholesterol in the endosomal membrane. This phenomenon, in turn, increases the number of ILVs inside MVBs. Decreased NPC1 triggers a low-rate endosome-to-ER cholesterol transport. Also, the down-regulation of the AKT/mTOR pathway suppresses the formation of autophagolysosomes. Schematic of monocyte activation, NF-κB accumulation, and secretion of proinflammatory cytokine in breast and gastric cancers (Lower left). Exos released from breast and gastric tumor cells increases THP-1 monocytes differentiating to mature macrophages. Proinflammatory cytokines such as TNF-a can activate STAT3 and TLRs in a MyD88-dependent manner. In inflamed cardiac tissue (Lower right), some factors such as angiotensin II, LPS, and hypoxic conditions increase the transfer of miR-155 from macrophage into Exos. Secretory Exos enter endothelial cells (ECs) and activate NF-κB, which is a key molecule between inflammation and cardiovascular disease. Synthesis and secretion of pro-inflammatory cytokines by NF-κB lead to hypertrophy and inflammation in the affected cardiac tissue. Created using CorelDRAW Graphics Suite Ver. 250.0.0.230.

inhibition/stimulation of TLRs. Under pro-inflammatory conditions, the activation of TLRs especially TLR-4 in the recruited immune cells can promote inflammation-induced angiogenesis.[117] The activation of TLR-4 leads to the activation and homing of certain immune cells such as lymphocytes into injured sites. Upon reaching the target sites, these cells can release several angiogenesis factors and inflammatory cytokines.[118] Notably, ECs like several cell types can express TLRs and these receptors are activated in response to damage signals and pathogens.[119] It was suggested that the activation of endothelial TLRs can be done in a paracrine manner via the interchange of Exos between the ECs and other cell types such as immune cells.[120] For instance, Dalvi et al claimed that LPS- and interferon alpha-primed monocytes secret Exos with distinct miRNA signatures that can promote the expression of adhesion molecules such as CCL2, ICAM-1, VCAM-1 on brain microvascular ECs via the activation of TLR-4 in a MyD88-dependent manner.[120] The suppression of Exo secretion in activated monocytes using GW4869 can reduce the inflammatory response in ECs (IL-1β↓, and IL-6↓). Thus, one could hypothesize that the TLR signaling axis has a critical role in the angiogenesis potential of ECs under pathological conditions. It should be noted that the activation of all TLR types does not yield similar angiogenesis potential. In an experiment conducted by Liotti and co-workers, they declared that the activation of TLR-7 in non-small cell lung cancer by imiquimod, a TLR-7 agonist, can reduce the release of VEGF-A, CXCL1, Ang-1, leading to the suppression of angiogenesis response.[121] Previously, it was indicated that the incubation of EPCs with LPS leads to the increase of Exo secretion via the elevation of intracellular $Ca^{2+}$in P3R-dependent endoplasmic reticulum manner and alteration of PMCA 1/4 expression after stimulation of TLR-4.[122] Heidarzadeh et al indicated that the incubation with LPS can affect the EPC survival rate in a dose-dependent manner in *in vitro* condition.[123] In LPS-treated EPCs, the levels of p-ERK1/2 and Erk1/2, NF-kB and TRIF were increased and led to the expression of Exo-related factors. Along with these changes, non-toxic levels of LPS can promote the angiogenesis potential of EPCs in mouse model of subcutaneously injected Matrigel plug.[123] Besides the critical role of the TLR signaling pathway in the production and release of Exos from ECs, Exos in turn promotes this signaling cascade in the recipient ECs. In an interesting study, Migneault and co-workers found that the exposure of ECs to serum-depleted conditions promotes apoptotic changes with simultaneous apoptotic Exos production. The attachment of apoptotic Exos to neighboring ECs induced cell migration and wound closure capacity while the angiogenesis potential was significantly diminished.[124] Molecular analysis showed that apoptotic Exo lumen contains specific genetic cargo with the potential to activate the NF-kB pathway via engaging TLRs, and RIG-I-like receptors.[124] In a similar study, it was shown that the treatment of rat aortic ECs with 100 ng/ml LPS promotes the generation and abscission of Exos compared to the non-treated ECs.[125] The uptake of Exos released from LPS-treated ECs was significantly higher in NR8383 alveolar macrophages compared to normal EC Exos. The internalization of pro-inflammatory Exos in macrophages induces M1-type phenotypes with the increase of CD86, and inducible nitric oxide synthase (iNOS). These features were orchestrated via the activation of TLR-4/NF-κB and phosphorylation of subunit p65.[125] It is also noteworthy to mention that human $CD54^{+}$monocyte microparticles harbor higher levels of pro-inflammatory cytokines like TNF-α, IL-6, and -8.[126] These particles can *per se* stimulates the production of Exos in brain hCMEC/D3 ECs.[126] Likewise, Treponema pallidum-treated dendritic cell Exos yielded similar outcomes on HUVECs via the activation of the TLR4/MyD88/NF-κB axis.[127] HUVECs exposed to dendritic cell Exos exhibited inflammatory phenotypes indicated with IL-1β, -6, and TNF-α. The inhibition of NF-κB with chemical inhibitor BAY11-7082 reduced these effects.[127] Emerging data have revealed the close relationship between the TLR signaling pathway and Exo release in endothelial lineage.[128] In mouse cardiac ECs, the activation of TLR-4 with bacterial LPS produced Exos with higher levels of Lamp-2, and $\alpha_v\beta_6$. Further uptake of $\alpha_v\beta_6$-loaded Exos by dendritic cells and macrophages can contribute to production of VEGF and angiogenesis response.[129] The phenomenon of Exo secretion and release is associated with the activation of several intracellular factors. In an experiment, it was shown that the release of Exo is dependent on certain miRNAs, especially miR-214. ECs needs miR-214 to release the Exos in which the inhibition of this factor blunt the angiogenesis capacity.[130] In several cancer cells, the activation of TLRs can induce specific miRNA types such as miR-214.[131] These data show the possible cross-talk between the TLR signaling pathway and Exo biogenesis mechanisms.

## Conclusion

TLR signaling pathway can affect several bioactivities in the host cells after being exposed to different danger signals. Based on emerging data, it was suggested that the TLR signaling pathway can affect the paracrine activity of cells via the release of Exos into the ECM. It is thought that several shared molecular mechanisms exist between the TLR signaling pathway and endosomal machinery. Thus, the modulation of TLRs can affect the Exo biogenesis and abscission in different cell types such as stem cells. Meanwhile, Exos can also mediate their effects on the target cells in part via the modulation of TLRs especially in immune cells. To be specific, these nano-sized particles can harbor several inflammatory cytokines that target PAMP receptors and TLR, leading to the stimulation of

## Review Highlights

**What is the current knowledge?**

- Exos participate in different cell functions and bioactivities related to several lineages.
- How and by which mechanisms, Exos exert their biological effects remain unknown.

**What is new here?**

- The close relationship between the Exo biogenesis, and regulation of inflammation was studied.

downstream molecules. Overall, continued and extensive research is needed to investigate the relationship between exosomes, inflammation, and tumors and how they mediate the inflammatory process. In the context of angiogenesis, the close relationship between the Exo, and TLRs is integral to EC activity. Thus, using certain therapeutic strategies, i.e., inhibition or stimulation of distinct TLR types, it is possible to control the blood supply to the affected sites.

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### Authors' Contribution

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### Competing Interests

The authors declare that they have no competing interests.

### Ethical Statement

This study was approved by the local ethics committee of Tabriz University of Medical Sciences under an ethical code of IR.TBZMED.VCR.REC.1399.477.

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