

Ectosomes as immunomodulators

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Abstract Considerable progress has been made in recognizing microvesicles as important mediators of intercellular communication rather than irrelevant cell debris. Microvesicles released by budding directly from the cell membrane surface (i.e., ectocytosis) either spontaneously or in response to various stimuli are called shed vesicles or ectosomes. Ectosomes are rightside-out vesicles with cytosolic content, and they expose phosphatidylserine in the outer leaflet of their membrane. Depending on their cellular origin, ectosomes have been associated with a broad spectrum of biological activities. In the light of recent findings, we now know that ectosomes derived from polymorphonuclear leukocytes, erythrocytes, platelets, and tumor cells have profound effects on the innate immune system, as well as on the induction of the adaptive immunity, globally reprogramming cells such as macrophages or dendritic cells toward an immunosuppressive and possibly tolerogenic phenotype. Although the effects observed in the circulation are mainly procoagulant and proinflammatory, ectosomes might be anti-inflammatory/immunosuppressive in local inflammation.

Keywords Ectosomes · Microparticles · Shed vesicles · Inflammation · Immunosuppression

Introduction

Over the last years, it became evident that communications between cells relied not only on soluble mediators or cell–cell contact, but also on the release of small vesicles by one cell that transport information to another cell, which might be in the vicinity or even at a distance. These vesicles are described in the literature by multiple names including microparticles (MP) or microvesicles. The messages transmitted involve proteins, lipids, and nucleic acids. This type of communication is not limited to multicellular organisms. MP have been found in the matrix of bacterial biofilms and to deliver signals critical for coordinating group behavior (quorum sensing) [1, 2]. The evident advantages of vesicles as messengers are: molecules inside the vesicles are protected from the environment and those expressed at the surface are in a conformation that allows multiple interactions, thus more information can be provided. For example, vesicles from platelets (PLT) provide the surface necessary to induce thrombosis. Other aspects include the transfer of molecules to the recipient cell, either on the cell surface (e.g., transfer of tissue factor from vesicles of monocytes to PLT) or that influence the expression of proteins (e.g., RNA transfer that allows the expression of a novel protein by the recipient cell).

Ectosomes—shed vesicles

Stein and Luzio coined the term *ectocytosis* to describe the complement-triggered shedding of rightside-out plasma membrane vesicles from the surface of polymorphonuclear

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leukocytes (PMNs) [3]. They wanted to clearly distinguish the mechanisms related to “ectocytosis” from those of exocytosis, which is used to describe the fusion of intracellular secretory vesicles with the cell membrane followed by the release of their content. Shed vesicles/ectosomes are budding directly from the cell membrane (Fig. 1) [4] and, for example, allow the removal of the C5b-9 attack complex from the cell surface so assuring protection against complement attack [3]. Such protective function has been shown for PMNs, oligodendrocytes, and even erythrocytes [5–7]. However, ectocytosis does not only correspond to the removal of the C5b-9 complex, but also allows a specific sorting of membrane proteins into the shed ectosomes. Enrichment in cholesterol and diacylglycerol in the ectosome membrane attests for a specific sorting of lipids as well.

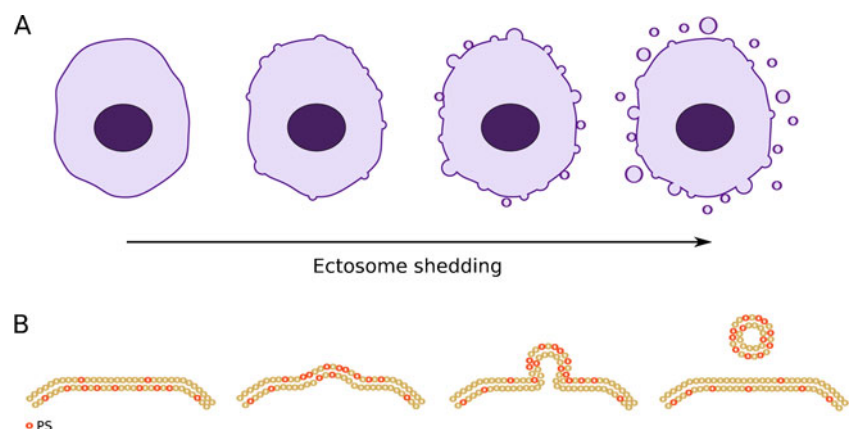
Although ectocytosis describes the same phenomenon in many eukaryotic cells, the stimuli inducing cell membrane budding can differ from one cell to another. Endothelial cells, erythrocytes, and PMNs release ectosomes when attacked by complement *in vitro* or exposed to other stimuli [3, 8–10]. Monocyte-ectocytosis is induced by bacterial cell wall components such as lipopolysaccharides (LPS), whereas PLT release ectosomes by activation through thrombin [11, 12]. Fibroblasts release ectosomes in response to stress relaxation when cultured in a three-dimensional collagen matrix [13]. Many cancerous cells have an activated phenotype with highly active ectocytosis in the absence of any stimulus [14, 15]. Indeed, the shedding of ectosomes is enhanced when cells are activated, ectocytosis is an ongoing process *in vivo* for many cells, and background levels of ectosomes originating from circulating and endothelial cells are found in blood [8, 9, 16, 17]. Physiological is the release of ectosomes by chondrocytes which leads to bone formation [16]. Ectosomes have often been associated with procoagulant and pro-inflammatory effects. Those derived from endothelial cells have been described to induce procoagulant activity in

monocytic cells [18], whereas ectosomes from PLT and monocytes were shown to directly promote hemostasis and induce inflammation by activating endothelial cells [12, 19]. Mackenzie et al. described the shedding of vesicles by monocytes as a mechanism to release rapidly IL-1 [20]. Ectosomes serve as intercellular protein carriers for tissue factor and the chemokine receptor CCR5 [21, 22]. The same group reported on the anti-inflammatory properties of ectosomes released by tumor cells, which suppressed B cell activation and reduced the activation of monocytes induced by LPS [23]. Finally, ectosomes have been proposed to be, besides apoptotic cells, an important source of auto-antigens [24], although in general apoptotic cells do not generate an immune response and are well known to down-regulate the inflammatory responses of monocytes and macrophages [25].

Ectosomes or soluble proteins?

Over the last 20 years, many reports described the release of soluble proteins from the cell surface by enzymatic cleavage. For instance, complement receptor 1 (CR1/CD35) is cleaved from the surface of leukocytes by elastolytic enzymes, and the large extracellular fragment of CR1 is found in human plasma [26]. However, the same CR1 found in urine was not a soluble molecule but bound to vesicles shed from glomerular epithelial cells (podocytes) [27–31]. Electron microscopic pictures of the renal glomerulus suggest that podocytes undergo under physiological conditions, a continuous process of vesiculation by way of ectocytosis, with the vesicles being released into the urinary space. Recently, Hara et al. confirmed by electron and immunoelectron microscopy using podocalyxin as biomarker of podocytes that apical cell membranes of podocytes were shed into urine [32]. They showed that podocalyxin-positive vesicles were similar in size to podocyte microvilli, and the electron microscopy examination revealed a tip vesiculation of microvilli. This explains

Fig. 1 Ectosomes are microvesicles budding from the cell membrane surface. **a** Small membrane vesicles shed by budding directly from the cell membrane are called ectosomes. **b** Detail of the shedding one ectosome. *PS* that is normally in the inner leaflet of the cell membrane flips to the outer membrane. The cell membrane that loses its asymmetry shed the formed vesicle/ectosome and returns to its normal state. However, the ectosome express *PS* on its surface



the release of CR1 bearing microvesicles in urine. While analyzing different biological fluids (bronchoalveolar and synovial fluids), it became evident that—as in urine and different from the plasma—only a limited amount of the total CR1 measured was soluble, the larger fraction being in a “membrane” form, later found to be ectosomes released by PMNs [33–38].

From the foregoing, it is evident that much attention has to be given to the analysis of molecules measured in biological fluids or in the supernatant of cell culture experiments. Centrifugation and ultracentrifugation of these fluids should always be performed so that vesicular bound and soluble molecules can be distinguished.

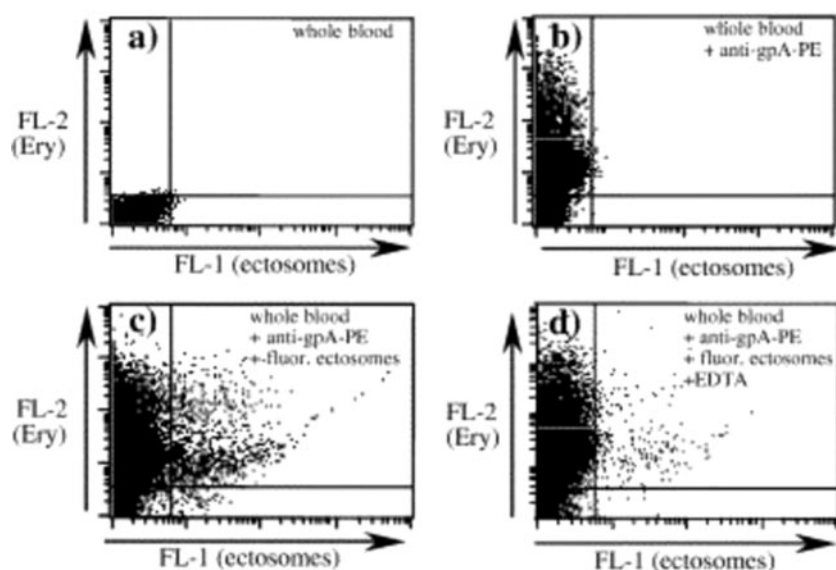
PMN-derived ectosomes (PMN-Ect)

In accordance with the observations made by Stein and Luzio [3], Hess et al. confirmed that the size, expression of selective markers, and presence of phosphatidylserine (PS) on the surface of vesicles released by PMNs activated with fMLP or C5a correspond to the definition of ectosomes [33]. By electron microscopy, it was possible to visualize the formation of buds on activated PMNs and the newly formed ectosomes, constituting a slightly heterogeneous population of vesicles with a diameter of approximately 50 to 200 nm [33, 39]. The pattern of biotinylated proteins between PMNs and PMN-Ect indicated a specific sorting of proteins into and out of PMN-Ect at the time of their formation. These ectosomes expressed a selective set of proteins originating not only from the cell membrane (selectins and integrins, complement regulators, HLA-1, Fc γ RIII, and

CD66b) but also from intracellular compartments (elastase, myeloperoxidase/MPO, matrix metalloproteinase-9, and proteinase 3). Thus, PMN-Ect do not correspond to inert particles, but to small vesicles with effector activities such as immune adherence and elastolysis [33, 39]. Particularly puzzling was the presence of enzymes (elastase, MPO) on the surface of PMN-Ect, which are normally localized in cellular granules. Using MPO deficient cells, it was possible to demonstrate that MPO was first released from activated cells in a soluble form, and then bound back to PMN-Ect as well as to by-standard cells [39, 40].

In vitro, PMN-Ect were found to specifically bind to endothelial cells and phagocytic cells (monocytes/macrophages and the monocytic THP-1 cell line). In addition, PMN-Ect were observed to bind specific proteins from plasma, in particular C1q, which is followed by complement activation and C3 fixation [39, 41]. In whole blood, PMN-Ect adhere to erythrocytes (complement-mediated immune adherence) similarly to immune complexes (Fig. 2) [41–44]. This immune adherence may allow PMN-Ect to be removed rapidly from the circulation by the fixed phagocytes of the liver, spleen, and bone marrow. Together with this rapid clearance, the adherence of ectosomes to erythrocytes may prevent binding to endothelial cells, thus avoiding harmful fixation in tissues, for instance glomeruli, as shown for apoptotic bodies and immune complexes [43, 45]. However, the surface expression of PS on PMN-Ect might have detrimental effects in the blood circulation due to interactions with the coagulation cascade. In vivo, PMN-Ect have been found to be associated with platelet MP, which are well-known catalyzers of thrombosis [46].

Fig. 2 Adherence of PMN-Ect to erythrocytes in whole blood. Representative FACScan dot plots showing the binding of PMN-Ect to anti-glycophorin A (anti-gpA)-PE-labeled erythrocytes (Ery) in hirudine whole blood. **a** Control fluorescence of whole blood in the absence of PMN-Ect. **b** Anti-gpA-PE-labeled erythrocytes in whole blood in the absence of PMN-Ect. **c** Anti-gpA-PE-labeled erythrocytes in whole blood incubated with fluorescent PMN-Ect. **d** Anti-gpA-PE-labeled erythrocytes in whole blood incubated with fluorescent PMN-Ect in the presence of EDTA, which blocks complement



PMN-Ect express PS in the outer leaflet of their plasma membrane similarly to apoptotic cells [39]. Of note, unlike normal cells, ectosomes cannot restore this membrane asymmetry over time [20, 39]. A detailed review on the loss of the physiological asymmetry and its consequences is given in another article of this issue [47]. The clearance of apoptotic cells by phagocytes is dependent on a series of interactions between the surface of the apoptotic cells and the phagocyte. In addition, a series of soluble bridging molecules are known to enhance this binding. One of these molecules is C1q, which binds first to apoptotic bodies, allowing them to be ingested more efficiently by macrophages [48–50]. In C1q deficient mice, apoptotic cells are cleared improperly and with delay, which may be responsible for autoimmunity [45]. As mentioned above, C1q also binds PMN-Ect [39], and hence C1q binding may enhance the uptake of these ectosomes by monocytes/macrophages. The deposition of C3 fragments might have similar effects.

The next question is: do PMN-Ect modulate macrophage function? Gasser et al. showed that tyrosine phosphorylation is enhanced in THP-1 cells after they have bound PMN-Ect, suggesting the activation of one or more signal transduction pathways [39]. In addition, when undifferentiated THP-1 cells are incubated with PMN-Ect for 48 h, they change their phenotype (down-regulation of CD14). Gasser et al. found also that PMN-Ect induce the release of TGF- β 1 by human monocyte-derived macrophages *in vitro* and are able to block macrophages' inflammatory response to zymosan A and LPS (down-regulation of IL-8, IL-10, and TNF α secretion). Ectosome-to-cell contact is sufficient for this immunomodulatory function, with PS expression playing a central role [51]. The intracellular signaling pathways involved in down-modulating macrophages have been explored recently. The importance of Mer receptor tyrosine kinase (MerTK) in the "tolerogenic" clearance of apoptotic cells by macrophages and DCs was shown in *in vivo* and *in vitro* studies [52–56]. Corresponding to these observations, Eken et al. demonstrated that one of the major mechanisms responsible for PMN-Ect-mediated down-modulation of macrophages is the interaction of PMN-Ect' PS with MerTK (Fig. 3) [57], possibly via GAS6 secreted by macrophages [58]. Upon their encounter, MerTK activates phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which in turn blocks NF κ B p65 translocation to the nucleus, and its phosphorylation thus opposes the biological effects of TLR2 activation. Consequently, the expression of key pro-inflammatory genes such as TNF α , IL-1 β , IL-6, IL-8, IL-10, and IL-12 and their protein release are inhibited [57]. However, besides PS, other molecules of the ectosomes might induce additional signaling pathways responsible for the modulation of the macrophage response (e.g., cell surface components or soluble factors released by the target cells).

PMN-Ect have also biological effects on the differentiation of human monocyte-derived dendritic cells (DCs) [59], hence on the immune response. When immature DCs are stimulated with LPS in presence of PMN-Ect, their morphology, phagocytic activity, expression of cell surface molecules (CD40, CD80, CD83, CD86, HLA-DP DQ DR, and CCR7), cytokine release (IL-8, IL-10, IL-12p70, TNF α , and TGF- β 1) and capacity to induce T cell proliferation are altered. Together, these results suggest that PMN-Ect might induce a tolerogenic phenotype in DCs, similarly to what has been described for apoptotic cells [25, 60].

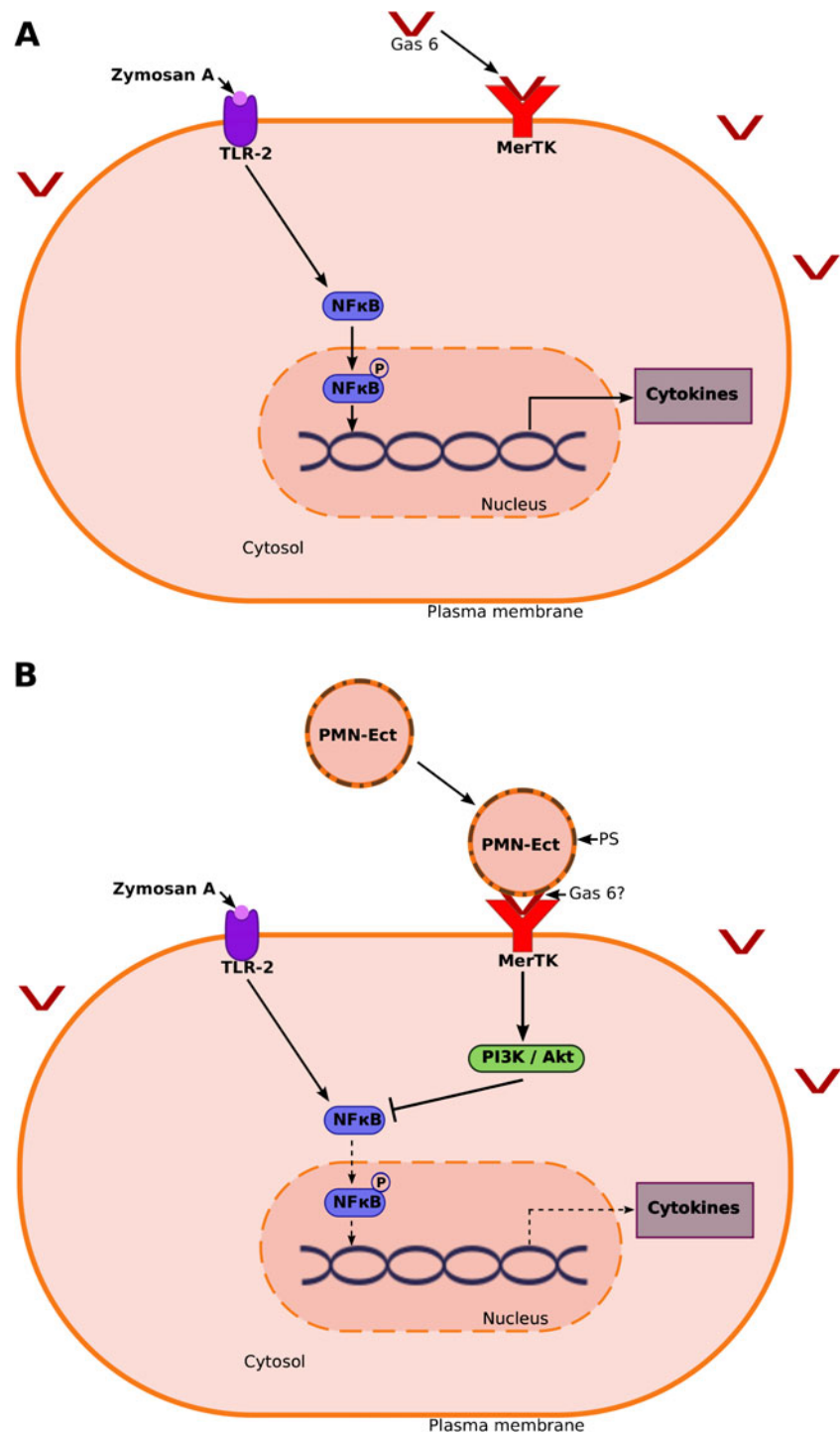
Taken together, these observations on macrophages and DCs indicate that PMN-Ect have unique characteristics that make them candidates for playing roles in inflammation and immunity.

Here, we would like to emphasize the complexity of the inflammatory process: on one hand, it needs to be amplified so as to trigger a specific immune response, and on the other hand, it has to limit excessive inflammation and prevent autoimmunity. At the site of injury, whether this injury is related to cell necrosis and/or infection, much phagocytic and inflammatory activity is needed. However, such local inflammation requires control as well and does not need systematically the stimulation of an acquired immune response. PMN-Ect are released at the early phase of PMN activation and have the property of early down-modulation, which in the local context may participate in the control of autoimmune responses, similar to the effect that has been suggested for apoptotic cells [25, 61–63]. However, a major difference between PMN-Ect and apoptotic cells is that PMN-Ect are involved very early in inflammation, a time point that might be crucial for determining later aspects of the cascade responsible for acquired immunity. Thus, PMN-Ect might not be involved only in terminating inflammation, but also in controlling the immune response.

It is noteworthy to highlight the effects of PMN-Ect on resting cells. Exposing immature DCs to PMN-Ect alter their morphology and their phagocytic activity [59], and in macrophages, PMN-Ect induce the phosphorylation of Akt [57]. Moreover, TGF- β 1 is rapidly released by both cell types upon PMN-Ect encounter [51, 59], an effect that merits further investigation. Such encounter does not induce apoptosis in the cells taking up ectosomes. To the contrary, PMN-Ect actively change the biological behavior of immature DCs and resting macrophages, suggesting that PMN-Ect reprogram these cells immediately even in absence of a specific stimulus.

In sum, PMNs, known to be central in inflammation, release potent anti-inflammatory and immunosuppressive effectors in the form of ectosomes at the earliest stage of inflammation, already providing a drive to its resolution [51, 57, 59].

Fig. 3 A schematic drawing of the pathways involved in the down-modulation of human macrophages by PMN-Ect. **a** Macrophages are activated by the ligation of zymosan A to TLR-2, which positively signals NF κ B translocation to the nucleus and its subsequent phosphorylation that leads to production of pro-inflammatory cytokines. **b** PMN-Ect bind via phosphatidylserine (PS) exposed in the outer layer of their membrane to Mer receptor tyrosine kinase (*MerTK*), possibly bridged by the ligand GAS6 released by the macrophages. Then, *MerTK* activates PI3K/Akt, which in turn inhibits NF κ B transactivation. Thus, the transcription and, consequently, the translation of pro-inflammatory cytokines are abrogated



Erythrocyte-derived ectosomes (E-Ect)

In vivo, circulating erythrocytes lose 20% of hemoglobin and membrane during aging by shedding vesicles. Hemoglobin-containing ectosomes are known to circulate in plasma [64]. Similar vesicles are produced during storage of erythrocytes. Blood transfusion has different biological effects in particular on the immune system. Post-operative

infections are increased in patients receiving blood transfusions, suggesting that components of blood are immunosuppressive [65–67]. Opelz et al. [68] reported many years ago that blood transfusion of cellular blood components improved renal allograft survival in transplant patients. The beneficial effect of blood transfusion on the number of rejection episodes and survival of transplanted kidneys was later confirmed [69, 70]. Other favorable effects of blood

transfusion have been described in autoimmune diseases, such as reduced recurrence of Crohn's disease [65]. Many authors have related the immunosuppressive properties of blood transfusion to one or the other component of blood, with particular attention to the transfused leukocytes [67]. For instance, an increased incidence of infections has been clearly associated with transfusion of non-leukocytes depleted blood. This might be due to the immunological reactions induced by "leukocyte transplantation" [71]. However, recent data suggest that the depletion of leukocytes is not enough to remove the negative biological properties of transfusion. The transfusion of leukocyte-depleted erythrocytes does not improve the survival in patients with colorectal cancer, and both types of transfusions are worse than no transfusion [72]. Recently, Koch et al. showed that in patients undergoing cardiac surgery, transfusion of red cells that had been stored for more than 2 weeks was associated with a significantly increased risk of post-operative complications as well as reduced short-term and long-term survival [73]. In addition, Atzil et al. have shown in a rat model that transfusion of fresh blood is less harmful than transfusion of stored blood in the context of progressing malignancies. Indeed, blood of either allogeneic or syngeneic origin stored for 9 days or longer significantly increased lung tumor retention in a storage time-dependent manner. Fresh blood, allogeneic or syngeneic, had no deleterious effect [74]. Less attention has been given to other components of stored blood with respect to immunosuppression. For instance, small vesicles/MP derived from erythrocytes during storage are abundant in packed erythrocytes, and their number increases with storage time [3, 64, 75, 76]. Thus, immunological properties of these vesicles are of particular interest.

Erythrocyte-derived MP released during storage show the characteristics of ectosomes, with a size between 50 and 500 nm, (Fig. 4) and the expression of erythrocyte surface

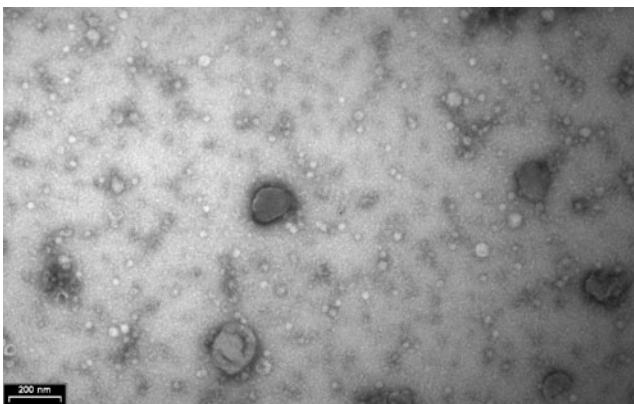


Fig. 4 Electron microscopy of E-Ect. Picture of a standard E-Ect preparation showing heterogeneity in size, which ranged from 50 to 500 nm. Size bar, 200 nm

marker and PS. The protein pattern of erythrocytes compared to that of E-Ect shows a specific sorting of proteins into and out of E-Ect at the time of their formation [77]. The work of Pascual et al. indicates that ATP-depleted erythrocytes, mimicking aging of erythrocytes, lose preferentially CR1 and DAF, which are both enriched in the ectosomes [38]. The expression of these complement regulators as well as CD59 might confer to E-Ect, a resistance to complement attack. In plasma, E-Ect bind C1q, which is followed by complement activation and C3 fixation. As already referred to, apoptotic cells as well as apoptotic bodies express PS and bind C1q, which ensures a more efficient phagocytosis by macrophages. The same accelerated phagocytosis might be valid for E-Ect as well [48, 49]. E-Ect exert an anti-inflammatory effect on activated macrophages leading to a significant decrease, almost abrogation of pro-inflammatory cytokines release (TNF α , IL-8 as well as IL-10). This inhibitory effect is immediate, a property shared with ectosomes of PMNs, and sustained for up to 24 h. Differently from ectosomes of PMNs, those of erythrocytes do not induce the release of TGF- β 1, whether the ectosomes are produced by aging (ATP depletion), storage, or Ca⁺⁺ ionophore.

It is to be expected that the properties of ectosomes are directly dependent on the originating cell. Thus, the biological difference observed for PMN- and E-Ect is not surprising. In addition, any cell may release different types of vesicles. For instance, PLT are known to release two types of MP: (1) vesicles of 100 nm to 1 μ m shed from their surface (equivalent to ectosomes) and (2) preformed exosomes released from multivesicular bodies measuring 40 to 100 nm in diameter [78]. The MP of PLT are described in the literature mainly as promoting coagulation via the exposure of PS. But does PS of platelet ectosomes induce inflammation as well or is it—as for other ectosomes and apoptotic cells—an element that controls inflammation?

Platelet-derived ectosomes (PLT-Ect)

PLT transfusions are necessary to prevent bleeding in thrombocytopenic patients. PLT concentrates used for transfusion are stored up to 4 days, time during which they shed large amounts of shed vesicles (ectosomes), which express a high concentration of CD61 and no CD63 [78]. During their storage time, they acquire proteins from plasma such as complement proteins C1q, factor H, and C3 fragments [79]. Interestingly, *in vitro*, PLT-Ect have similar effects on macrophages than those of PMNs; they induce an immediate release of TGF- β 1 and they reduce the pro-inflammatory cytokine release. Thus, it is of interest to realize that PLT-Ect have many faces including procoagu-

lant activities, but also others that may dampen inflammation under specific circumstances.

The fate of ectosomes

To date, little attention has been given to the fate of ectosomes, once they have reached their target. First, they might just remain adherent to the target cells, as evidently observed for the binding of ectosomes to erythrocytes in the presence of plasma. Second, they might be integrated into the membrane by fusion as described by Del Conde et al. [80]. These authors have nicely showed that tissue factor on monocyte/macrophage-derived microvesicles do not only bind activated PLT but fuse with them through a mechanism involving P-selectin glycoprotein ligand-1 on the microvesicles and P-selectin and PS on the PLT. This fusion/integration transfers both proteins and lipid to the platelet membrane and allows the content to be put in immediate contact with the cytoplasm of the target cell. Third, ectosomal proteins and lipids that have been ingested by macrophages might be re-expressed at the cell surface after having recycled in the cell. Whereas there is evidence for ingestion, the re-expression has been postulated but not demonstrated. These three mechanisms are not exclusive, and depending on the circumstances, the type of ectosomes and target cell, one or the other might be preferred. It is evident that depending on the type of incorporation, transferred molecules might or might not transfer signaling properties as well.

Concluding remarks

The biological roles of ectosomes might depend not only on their intrinsic properties, but also on the site of their formation. Evidently the high expression of PS on ectosomes of PMNs, erythrocytes and PLT in the intravascular compartment might help to promote a prothrombotic state with all its ensuing consequences. The anti-inflammatory properties described in this review may thus be well masked, but may, on the other hand, provide some limitation to the inflammatory process induced by the coagulation and complement cascade. In tissues, particularly for PMN-Ect, the situation will be very different and such ectosomes are more likely to encounter macrophages or DCs and limit their inflammatory/immune response activities, with a resulting dominating anti-inflammatory effect.

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Conflict of interest The authors declare that they have no conflict of interest.

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