# Communicating with the dead: lipids, lipid-mediators and extracellular vesicles.

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Abstract:
**Apoptosis is a key event in the control of inflammation. However, for this to be successful, dying cells must efficiently and effectively communicate their presence to phagocytes to ensure timely removal of dying cells. Here we consider apoptotic cell-derived extracellular vesicles (ACdEV) and the role of contained lipids and lipid mediators in ensuring effective control of inflammation. We discuss key outstanding issues in the study of cell death and cell communication, and introduce the concept of the *'active extracellular vesicle'* as a metabolically-active and potentially changing intercellular communicator.**

Communication between cells is an essential feature of multicellular organisms and extends to communication between dying cells and their viable counterparts. The mediators of communication are complex and varied, and a role for vesicles and small lipid mediators has become more appreciated over recent years. The study of this communication with dying cells has led to a clear appreciation that cell death underpins many physiological and pathophysiological processes including immune system responses to challenge.

Inflammation is a key component of the innate immune system and, whilst protective in the initial stages of an immune challenge, it can drive significant disease if controlled ineffectively. The processes driving inflammation have been defined in some significant detail, yet those processes that resolve the inflammation remain relatively ill-defined. However, cell death (apoptosis) has emerged as a key physiological programme that is central to the control of inflammation, and as a process for disposing of unwanted cells *in vivo* whether they be effete, damaged, infected or simply surplus to requirements, as may be the case towards the end of an inflammatory response.

Given the importance of this physiological role for cell death, the apoptosis programme is directed towards ensuring dying cells communicate their presence and become modified such that local and recruited cells with phagocytic capacity (e.g. macrophages) can effectively remove the cell corpses. The processes by which phagocytes are recruited to sites of cell death are becoming clear and it is apparent that the process is more than simple 'burial' of cell corpses, as it includes profound immunomodulatory effects. Furthermore, those processes that act to initiate inflammation appear to be crucial to driving those processes to resolve inflammation, demonstrating the highly coordinated and orchestrated nature of inflammation.

Here we recap the processes by which dying cells communicate their presence to phagocytes. We focus on lipid mediators and extracellular vesicles, and highlight some challenges remaining in defining our understanding of apoptotic cell-phagocyte communication.

**Cell death, inflammation and disease**

The critical importance of clearance of dying cells for the control of inflammation is highlighted in those diseases where the process fails, diseases chronically associated with ageing. Most notably this occurs in autoimmunity (e.g. SLE) where complement deficiencies (e.g. $C1q^{-/-}$) results in defective corpse clearance and shows a direct

causal link with defective apoptotic cell removal and inflammatory sequelae(1-3). However, there are other diseases where inflammation is central to the aetiology of the pathology and in these diseases, cell death plays a pivotal role. One such example is atherosclerosis where an inflammatory site, initiated by fatty deposition in the arterial wall, fails to resolve and becomes chronic. The lipid-laden environment is toxic to infiltrating leukocytes such that apoptosis is a key feature of developing plaques and, despite recruitment of monocytes and macrophages to clear dying cells, the inflammation fails to resolve and progresses (4, 5). In this case monocyte recruitment is driving disease as, once recruited, the monocytes are exposed to the toxic environment and, importantly, become trapped, fail to emigrate and die (6). Thus, this represents a clear therapeutic target where improved understanding of the mechanisms by which phagocytes are recruited to sites of cell death, may allow for targeted intervention to halt, if not reverse, the pathological process.

A further important disease target is cancer where interesting observations over many years have highlighted that cell death within a tumour may not be beneficial, as tumours with high levels of apoptosis appear most aggressive (reviewed in (7)). Similarly, those tumours with the greatest number of macrophages are also associated with poor prognoses. Whilst these observations seem counterintuitive, recent work has demonstrated that, at least in lymphoma, cell death within a sub-population of tumour cells drives macrophage recruitment (8, 9) but, rather than being a part of an effective anti-tumour response, the macrophages become pro-tumour in their phenotype as a result of dying tumour cells driving an M2 skew in the macrophages. Thus again, this represents a further pathology where inhibition of phagocyte recruitment towards dying cells may well be a key novel approach to preventing disease progression.

**Cell recruitment and control of inflammation: lipid mediators and beyond.**

The immune system response to tissue damage comprises carefully coordinated interactions between a range of immune and non-immune cells. The inflammatory response, initiated by local resident cells, leads to local vascular changes enabling leukocyte, initially neutrophil, recruitment (10). For the inflammation to resolve (i.e. “switch off”) it is essential that further cell recruitment is halted and neutrophils (PMN) are removed from the local inflamed site (11). This carefully programmed process involves reduction of neutrophil infiltration, migration of macrophages to the inflamed tissue, uptake of apoptotic neutrophils by the macrophages and their removal via the lymphatics.

The well-timed cellular movement seen throughout the inflammatory process is orchestrated by a series of chemical signals that generate a chemical gradient, “call” for leukocyte movement and/or block it (12). The entire acute inflammatory response is governed by balance of different signals, some of microbial origin, while others are locally biosynthesised at the site of tissue injury.

Lipid mediators play an important role in the acute inflammatory response (13) and the migration of neutrophils is initiated by the prostaglandins (PG) and leukotrienes (LT) - cyclooxygenase (COX) metabolites of arachidonic acid. Elevated levels of PG and LT contribute to chronic inflammation, thus both lipid metabolites are classically considered pro-inflammatory. However, $PGE_2$ and $PGD_2$ can also mediate a “class switch” of lipid mediators of inflammation where the balance of mediators is tipped in

favour of anti-inflammatory/pro-resolution mediators (14, 15). For example, $PGD_2$ and $PGJ_2$ have high affinity for a G-protein coupled receptor PD1 (16), thereby promoting resolution of inflammation (17). Additionally, $PGE_2$ switches on the transcription of lipoxygenases (e.g. 12-LOX and 15-LOX), family of enzymes required for a biosynthesis of small specialist pro-resolving lipid mediators (SPM) such as lipoxins (LX; (18)), protectins (PD; (19)), maresins (Mar; (20)) and resolvins (Rv; (19, 21).

These SPM are dual-acting metabolites: LX and Rv block PMN infiltration to the sites of injury (anti-inflammatory action) while promoting the recruitment of non-inflammatory monocytes, while LXs, Rv, Mar and PD stimulate phagocytosis of apoptotic neutrophils (i.e. efferocytosis) and cellular debris by pro-resolving macrophages (pro-resolution action) (22, 23). Looking from this novel angle, inflammation is actively regulated, both temporally and spatially, from its onset towards the resolution phase. Thus, loss or inhibition of any of the cell-receptors for lipid metabolites or their deficiency can lead to the resolution failure and lead to the chronic inflammation. However, it is important to note that metabolites of $PGE_2$ and $PGD_2$ (e.g. 15d-$PGJ_2$) alone can trigger the resolution phase and activate tissue remodelling without the "class switch" in eicosanoid production (24).

Clearly a key part of the resolution phase of inflammation is the 'sensing' of dying leukocytes followed by their removal. A number of mechanisms by which phagocytes are recruited to dying cells have been proposed (reviewed in detail (25)). Besides lipid mediators, these include the release of so-called 'find me' signals such as released nucleotides (ATP, UTP) (26, 27), chemokines (CX3CL1) (28) and lipids (lysophosphatidylcholine (29, 30) and sphingosine-1-phosphate (31)) of which at least some exert their pro-migratory effects through ligation of GPCR. However, the involvement of extracellular vesicles (EV) is becoming increasingly recognised (32) (33) and we propose the term 'apoptotic cell-derived extracellular vesicles (ACdEV) to cover this complex population of EV. These ACdEV may derive from a range of sub-cellular sources (e.g. plasma membrane, multivesicular bodies). Whilst these 'find me' signals may help promote recruitment of those phagocytes to help remove dying cells and thus resolve inflammation, another key event in the control of inflammation is to halt influx of other inflammatory cells. In this regard, 'keep out' signals have also been reported to be released from dying cells and these can reduce granulocyte recruitment (34).

**Extracellular Vesicles: a complex functional mediator**

The involvement of EV in the resolution phase of inflammation provides an added complexity to the process by both the multi-molecular composition of these EV factors and the great heterogeneity within the EV population. EV have been considered to be 'waste bags' that assist removal of unwanted material from the cells of origin(35, 36). Whilst this may be a valid function of EV, it is becoming increasingly clear that EV are mediators of intercellular communication and material exchange, and active loading of factors is likely (37).

EV are actively secreted from healthy, stressed and diseased, viable and apoptotic cells through three discrete biogenesis pathways (Figure 1A). A key challenge in the field of EV is to differentiate different sub-populations (based on either on physical or functional characteristics) of EV from within the highly heterogeneous population. This has, perhaps unwisely though understandably, often focused on size of EV, as a

feature that can be measured with relative ease using direct measures (e.g. using tunable-resistive pulse sensing) or indirect measures (e.g. particle tracking analysis or dynamic light-scattering).

Exosomes (~30-150 nm) are formed via an endosomal pathway through an inward budding to form multivesicular bodies (MVB) that may fuse with the plasma membrane to release contained EV. Microvesicles/microparticles (~100-1000 nm) are released by budding from the plasma membrane, while apoptotic bodies (50+ nm) are released from apoptotic cells (Figure 1A) though remarkably little is known of the formation and release of apoptotic bodies. Regardless of the mode of biogenesis, the EV population is a complex entity, rich in proteins, lipids, DNA, RNA, mRNA and miRNA, surrounded by a phospholipid bilayer (Figure 1B). EV mediate intercellular communication by delivering their cargo (which may be integral to the membrane, intra- or extra-luminal) to neighbouring cells via different mechanisms, including phagocytosis, membrane fusion and endocytosis (Figure 1C) (38, 39). Thus, EV can trigger cellular responses by ligand-receptor interaction or delivery of agents to the cytoplasm of recipient cells. It remains to be formally reported if ACdEV comprise EV from each of these three divisions (exosome, microvesicle/particle, apoptotic body) though it seems likely that as cells progress through different phases of apoptosis, the composition of the ACdEV population will differ (e.g. as cells move through stress to apoptosis commitment).

There is an increasing body of work that demonstrates the importance of EV in cross-talk with the innate immune system but, from an apoptosis perspective, the detail is limited. Whilst ACdEV are known to recruit phagocytes, only CX3CL1 (28) and ICAM-3 (33) have been identified as key molecular promoters of this recruitment. CX3CL1 can function as both chemokine (38) and adhesion molecule (40) whilst ICAM-3 is best known for its role as an adhesion molecule mediating initiation of immune responses (41) or tethering of apoptotic cells to phagocytes (33, 42, 43). Given the 'usual' jobs of these molecules, it seems likely that they support the association of EV with phagocytes and a range of other molecules may then be responsible for immune-modulatory effects.

A key component of EV is phospholipid and exposed phosphatidylserine (PS) is well established on apoptotic cells (44, 45) and EV. Given the immune modulating role of PS on AC (promoting AC uptake (46, 47) and driving TGF-$\beta$1 and IL10 production from macrophages (48, 49)), it seems likely that this exposed PS will also be an active component of ACdEV. Oxidation of exposed PS has also been shown to modulate apoptotic cell clearance (50, 51) though the importance of oxidised phospholipids in EV function are yet to be considered.

Whilst phospholipids are themselves essential for the structure of EV, the catabolic products of phospholipid metabolism may also be crucial, functionally-active components, such as COX- and LOX-derived lipid mediators of inflammation. Previous work has shown that microparticles derived from activated neutrophils carry LtB4, PD1, and primary products of enzymatic PUFA oxidation, namely 4-, 7- 14- and 17-hydroxy-docosaenoic acid, 5-, 12- and 15-hydroxy-arachidonic acid, 5,15-dihydroxy-arachidonic acid, 5-, 12-, 15- and 18-hydroxy-eicosapentaenoic acid and 5,15-dihydroxy-eicosapentaenoic acid (22). These primary LOX metabolites serve as direct precursors to the pro-inflammatory, anti-inflammatory and pro-resolving lipid mediators. Furthermore, EV uptake by M1 (inflammatory) macrophages changed the

lipid metabolite signature of M1 macrophage towards M1 phenotype. However, these studies have tended to focus on EV from activated PMN in 'augmented' culture conditions to maximise SPM release. They also focused on large EV (i.e. microvesicles) and so questions remain as to the lipid mediator carriage within smaller, more-diffusible (i.e. distant-acting) ACdEV and also from a greater range of dying leukocytes and non-leukocytes. This work is currently underway in our laboratory.

**Towards Unlocking Apoptotic cell-derived Extracellular Vesicles**

There is much still to discover in relation to EV in general, and those from apoptotic cells, in terms of biosynthesis, structure and function. From a functional perspective, it is clear that ACdEV and EV in general carry many molecules and often studies seek to identify the function of a single molecule within the complex EV environment. How these different molecular species fit together to communicate is a key challenge.

Our initial observations suggest that ACdEV carry a range of lipid mediators both pro- and anti-inflammatory. These results are in line with previous observations from activated neutrophil EV, but the fine blend of lipid mediators is yet to be defined fully in ACdEV. Key questions arise from this work, questions that are currently being addressed in our laboratory. Is there a lipid mediator signature that defines ACdEV and their pro-resolution function? Or is it simply that, whilst the specific lipid mediators within ACdEV from different cells may change, they are functionally conserved? How might the function of ACdEV lipid mediators change when the EV environment (e.g. proteome) may also change?

The process of apoptosis is broadly accepted to result in rapid removal of dying cells to prevent secondary necrosis and the inflammatory sequelae that would follow. Thus, it is possible that ACdEV may change in their composition and function throughout the apoptosis programme to effect different functional effects in the targets cells that receive these EV. For example, annexin A1 has been shown to promote migration of monocytes in response to secondary necrosis (i.e. necrosis following apoptosis) through ADAM10 processing of annexin 1 for release (50). This work raises the likely possibility that attractants will change throughout the apoptosis programme.

Remarkably little is known of the mechanisms that result in EV from apoptotic cells. It is often assumed that AC produce 'apoptotic bodies' from membrane blebs that are released and these have long been known to be released in a manner dependent upon cytoskeletal organisation (52) and under the control of Bcl-2 and caspases (53). These apoptotic bodies are widely reported to be large and simply smaller 'samples' of AC, with the same components, that are more easily phagocytosed. However, in the original seminal paper introducing apoptosis, it was clear that apoptotic bodies were of "greatly varying size…with only the largest discernible by light microscopy…. with smaller bodies dispersing from the site of origin" (54). From a functional perspective, it seems reasonable that these smaller bodies may be most effective in recruitment of distant phagocytes.

The characteristic plasma membrane physical changes seen in apoptosis, that begin before PS redistribution, may vary in different cells (adherent versus non-adherent) (37) and this may underpin different EV release kinetics from the plasma membrane. The overall process appears dependent on myosin light chain phosphorylation and

Rock I which promotes membrane blebbing (55-57). Over recent years, studies of the generation of EV from the plasma membrane of apoptotic cells (i.e. apoptotic bodies') has shed light on their release and there are clear morphological phases in addition to the classical membrane blebbing which has been proposed to be insufficient for EV release (58). In addition to blebbing, apoptotic cell-membrane protrusions have been proposed to be important in apoptotic body release from dying cells through a variety of membrane protrusions e.g. microtubule spikes (56), thin membrane protrusions that link membrane blebs ('apoptopodia') (59) and beaded apoptopodia (58). Release of plasma membrane-derived EV has been shown to be controlled via Pannexin I (59).However, EV are derived from various cellular sources and the contribution of EV from multivesicular bodies ('exosomes') or plasma membrane ('microvesicles') to the population of ACdEV is currently not known and is under investigation.

Whether ACdEV are all derived from the plasma membrane or whether different size EV arise from different cellular compartments remains to be elucidated, as does the composition and function of these different EV. Whilst it is possible that all ACdEV are broadly similar in their structure and function, it seems most likely that different sub-populations of the heterogeneous EV mix are structurally and functionally distinct. Our preliminary studies suggest that differences in function between ACdEV from different phases of apoptosis may differ in their activity. Perhaps small EV, that disperse easily from their site of origin, are mostly supportive of phagocyte recruitment whilst larger EV are more supportive of immunomodulation. It is well established that AC can drive pro-resolution phenotypes in phagocytes (48, 49). It remains to be seen if small vesicles have the same effect.

## EV – challenges & future directions

EV present a number of key challenges in elucidating their biological function. Their complexity and multiple cargo suggest that the net effect of any EV will be result of the balance of mediators that are carried (e.g. pro-inflammatory versus pro-resolution mediators). The structure/function analyses of EV are compounded further by the low abundance/high specific activity of the mediators and the small size of the ACdEV themselves. In order to simplify the heterogeneous nature of ACdEV we typically focus on sub-micron EV sizes and exclude larger vesicles so as not to skew our understanding only to those larger EV. In undertaking these studies, it is clear that large numbers of EV are required to identify lipid mediators in small quantities. This may raise concern with some over physiological relevance. However, a new level of complexity that remains to be addressed is the 'multiple waves' of EV release that may occur (figure 2). Our current studies are focused on ACdEV release from dying cells and there may be 'waves' of EV produced at different stages of apoptosis with profoundly different functional effects. However, it is entirely plausible that *in vivo*, it is the consequent and subsequent immune-response EV that may form a bigger, more active and perhaps most significant EV wave (i.e. ACdEV may recruit phagocytes which themselves release further functional EV to amplify responses). Thus, waves of lipid mediators may be released at different relative times within the inflammatory process and this may be critical to effective resolution of inflammation.

Additionally, a recently identified and exciting development in EV biology was the identification of the carriage of active-enzymes (60, 61). This raises the possibility that ACdEV may well be metabolically-active compartments that carry enzymes,

substrates, intermediates and final products from parent cells to recipient cells. It is likely that EV in general will carry enzymes following the 'sampling' of parent cells during EV generation though the importance of such packaged enzymes remains to be studied. Indeed, many proteomic studies have reported detection of enzyme presence (e.g. (62)) though enzyme activity *in situ* are rarely reported. Recently, the surface of exosomes has been reported to contain active proteases and glycosidases that may be responsible for remodelling of extracellular matrix (ECM) (63), with EV being proposed as active components of the ECM (64). In relation to inflammatory control, EV have been suggested to promote inter-cellular transfer of phospholipases and prostaglandins (65). However, in the case of ACdEV and lipid-mediators of inflammation, ACdEV which carry phospholipid substrates may also be considered *'active EV'* through the carriage of the machinery to generate further mediators through the action of phospholipases, cyclooxygenases and lipoxygenases. Thus, we propose that 'active EV' may be considered those EV that carry *in situ* enzymatic activity, rather than being shuttles for enzymes to recipient cells. Consequently 'active EV' may be constantly-evolving, complex mediators e.g. of inflammation and control. Perhaps ACdEV, following release, become more potently attractive to phagocytes and more immunomodulatory the further (in distance and time) that they travel *in vivo*. This would enable rapid release of EV in the apoptosis programme without the need for delay whilst enzymes and mediators are produced, enabling a time efficient release ACdEV that become more functional as the 'mature' on their travels. Whilst such variation adds more complexity to the analytical approaches of EV structure/function studies with EV perhaps constituting a 'moving target', it raises exciting potential insight to the function of the emerging field of EV and how EV may be functionally 'tailored'.

Might these processes change throughout ageing, and might they underpin healthy ageing? Certainly, innate immune function is known to alter with age and this may critically alter the resolution of inflammation(66). Such a change may contribute to so called 'inflammaging' and a clear view on what changes occur in ACdEV across the life course may help us to define targeted strategies for modulating the undesirable inflammatory consequences of ageing. Poorly controlled innate immune responses may underpin a number of age-associated pathologies (e.g. cancer, autoimmunity and CVD) and undesirable consequences of ageing (e.g. poor vaccine sero-conversion amongst the elderly)(67). This is a key area for future study. Dietary supplementation of PUFA has yet to show robust beneficial effects on disease but perhaps an effect on inflammatory ageing may be worthy of study, given the essential role for PUFA in the generation of specialist pro-resolving mediators.

**Figure legends:**

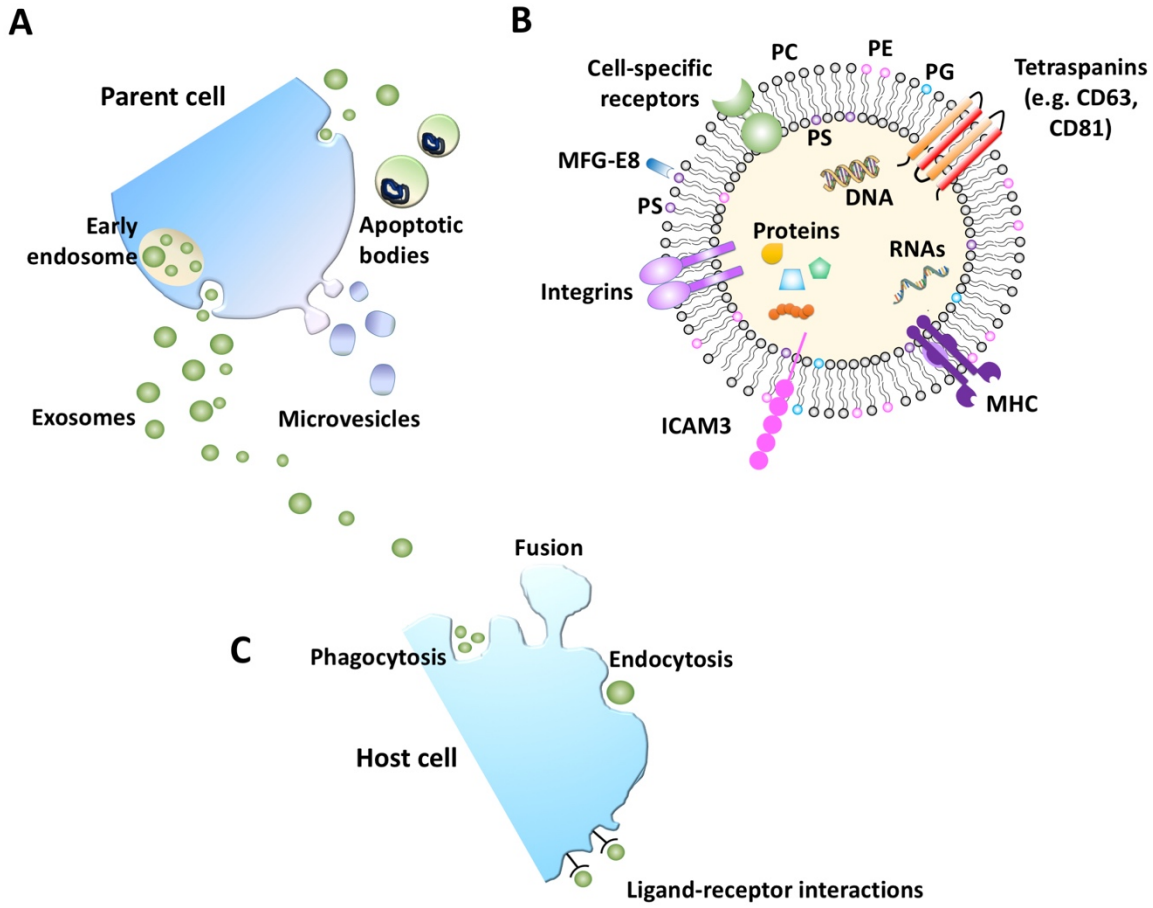


**Figure 1:** A. generation of EV from the endosomal compartment (exosomes) or the plasma membrane (microvesicles/microparticles; apoptotic bodies. B. Schematic diagram of the structure of an EV showing redistributed phospholipids (e.g. PS: phosphatidylserine; PE: phosphatidylethanolamine) and important EV proteome constituents (e.g. tetraspanins, adhesion molecules (e.g. ICAM-3), soluble opsonins (e.g. MFG-E8)). C. mechanisms of delivery of EV to recipient cells including phagocytosis, endocytosis, receptor-mediated uptake and membrane fusion).

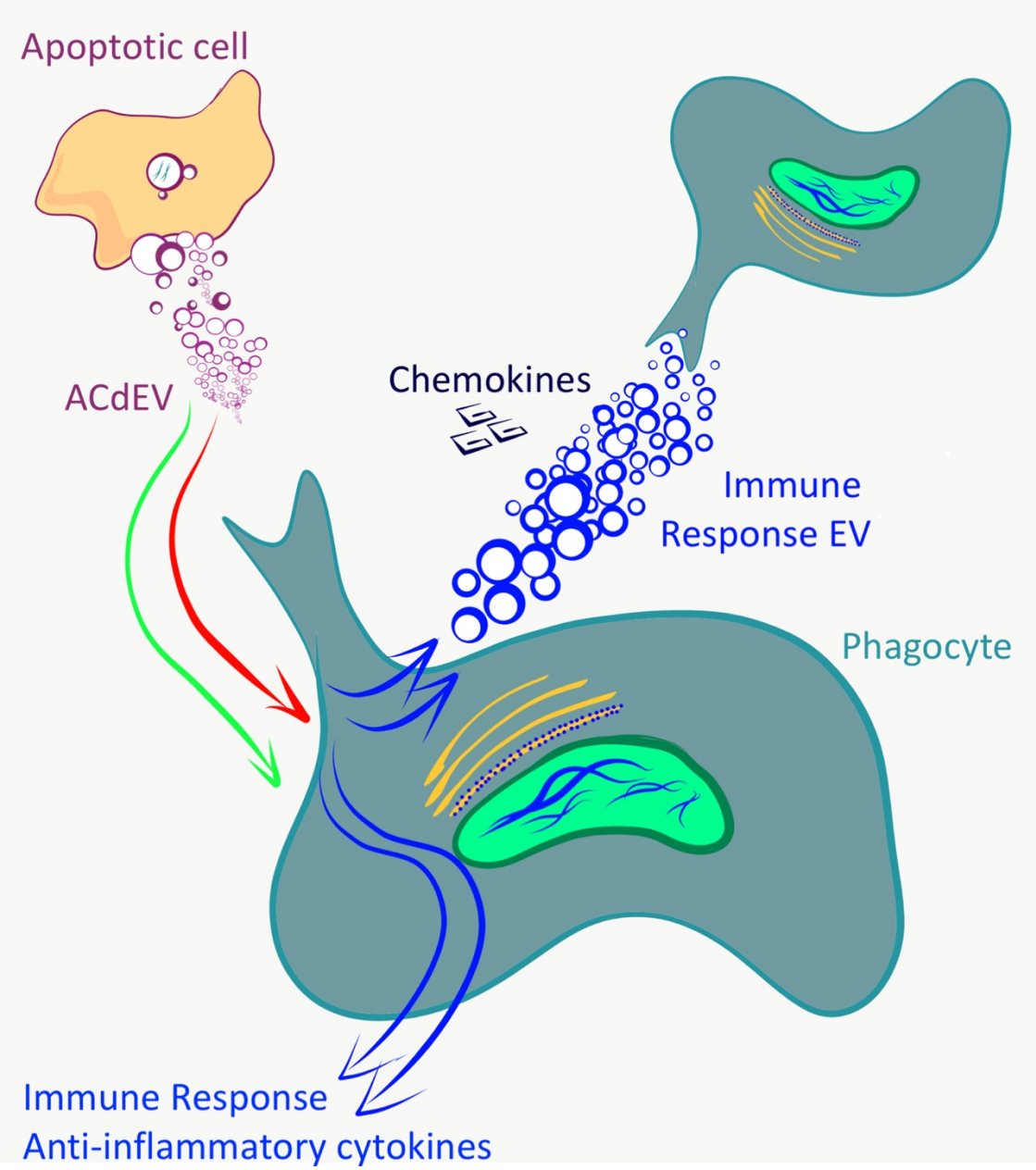


**Figure 2:** Putative Immune modulation by apoptotic cell-derived EV. ACdEV released from cells undergoing apoptosis can recruit 'first responder' phagocytes towards the dying cells to promote clearance. These ACdEV may change over the course of apoptosis to generate different 'waves' of ACdEV (as depicted by the red and green arrows). This pro-resolution event may be supported through the responses of those first recruited phagocytes who, through the production of anti-inflammatory cytokines and additional 'immune response' EV may modulate the function of an additional 'wave' of pro-resolution cells. Such a strategy may help to amplify the immune-modulating response to dying cells, as seen in inflammation. It is possible that different waves of ACdEV may induce different waves of immune responses via both cytokines and immune response EV (depicted by multiple blue arrows) and thus different outcomes (e.g. pro-resolution versus pro-inflammation).

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